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.....OF COWPEA MOSAIC VIRUS.....

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Isolation and Characterisation of Mutants of Cowpea Mosaic Virus.

Claire Louise Lesley Holness

A thesis submitted in accordance with the regulations of the University of Warwick for the degree of Doctor of Philosophy.

The research for this thesis was conducted at:
The Department of Biological Sciences, University of Warwick and at
The Department of Virus Research, The John Innes Institute, Norwich.

September 1989

For Maureen and Dennis Holness, Marianne-Emma and Norman Broddle.

C.L.L. HOLNESS. PhD (Warwick University) 1989

ISOLATION AND CHARACTERISATION OF MUTANTS OF COWPEA MOSAIC VIRUS.

ABSTRACT.

A nitrous acid-induced, temperature sensitive mutant of cowpea mosaic virus (CPMV) known as 8-14, (Evans 1985, *Virology* 1985, **141**, 275-282), was characterised. The phenotypic defect in 8-14 was shown not to affect translation of the RNA or the first proteolytic cleavage of the B RNA-encoded polyprotein. The defect is probably at the level of genome replication. The technique of two dimensional RNA fingerprinting showed the mutant genome to be similar to the parental wild-type but did not resolve the genetic alteration(s) specific for the mutation.

The mechanism of CPMV translation was investigated by site-directed mutagenesis of a full-length cDNA clone of CPMV M RNA from which infectious RNA could be generated by in vitro transcription. The results obtained confirm the AUG at position 161 is used to direct the synthesis of the 105K protein in vitro. The detection of a 58K protein in infected protoplasts suggests that it is also used in vivo. The synthesis of the 95K protein can be initiated from either of the AUGs at positions 512 and 524. Synthesis of this protein is not essential for CPMV replication in protoplasts.

Several deletion mutations were created in the M RNA cDNA clone in order to determine the regions of M RNA essential for replication of M RNA. Analysis of one mutant indicated that sequences between 1446 and 1620 are probably not required for replicase recognition. However, the accumulation of this mutant in protoplasts was reduced, presumably as a result of lack of encapsidation of the RNA as this mutant is thought not to synthesise functional coat protein. Data from several mutants showed that alterations of M RNA around nucleotides 161 and 189 prevent transcript accumulation in protoplasts possibly owing to a severe reduction in replicability of the input RNA.

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ABBREVIATIONS

Å	Angstrom
ALMV	alfalfa mosaic virus
BMV	brome mosaic virus
BNYVV	beet necrotic yellow vein virus
B RNA	CPMV bottom component RNA
cDNA	complementary deoxyribonucleic acid
CPMV	cowpea mosaic virus
DI RNA	defective interfering RNA
DTT	dithiothreitol
E.coli	Escherichia coli
FLT	full-length transcripts
K	apparent molecular weight in kDa
kDa	molecular weight (dalton [d] x 10^3)
LDT	linear DNA templates
min	minute
Mr	molecular weight (dalton [d] x 10^3)
mRNA	messenger RNA
M RNA	CPMV middle component RNA
Na_2EDTA	ethylenediaminetetra-acetate disodium salt
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
TBSV	tomato bushy stunt virus
TCV	turnip crinkle virus
TMV	tobacco mosaic virus
Tris	Tris (hydroxymethyl)aminomethane
TRV	tobacco rattle virus
ts	temperature sensitive
ug	micro gram
ul	micro litre

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DECLARATION.

I hereby declare that this thesis is the result of my own work and investigation. It has not already been accepted in substance for any degree and is not being currently submitted in candidature for any degree.

Claire L. L. Holness.

Chapter 1. General Introduction.

1.1 Comoviruses.

The comovirus group of plant viruses derives its name from the type member, cowpea mosaic virus (CPMV). Members of the comovirus group are serologically related and share common characteristics at the molecular, structural and biological levels. A definitive list of group members is not possible to compile since, as more information becomes available, some of the viruses may prove to be more appropriately considered as strains of others. The following fourteen viruses are currently classified as distinct comoviruses: cowpea mosaic virus, andean potato mottle virus, bean pod mottle virus, bean rugose mosaic virus, broad bean stain virus, broad bean true mosaic virus, cowpea severe mosaic virus, glycine mosaic virus, pea mild mosaic virus, quail pea mosaic virus, radish mosaic virus, red clover mottle virus, squash mosaic virus and Ullucus virus C (Bruening, 1978; Goldbach and van Kammen, 1985). Three other viruses; bean curly dwarf mosaic virus, virus de la mosaic da la féve and pea green mottle virus were formerly regarded as separate members of the group but have been reclassified, the latter two viruses now being considered as strains of broad bean stain virus and bean curly dwarf mosaic virus a strain of quail pea mosaic virus (Stace-Smith, 1981).

Biologically, comoviruses are characterised by having a narrow host range, leaf-feeding beetles as vectors, a low level of seed transmission and ease of sap transmission. Leguminous plants are infected predominantly by all but four comoviruses. Radish mosaic virus, squash mosaic virus, andean potato mottle virus and Ullucus virus C usually infect plants within the families Cruciferae, Cucurbitaceae, Solanaceae and Basellaceae respectively. As a result of the narrow

host range, there is essentially a single disease associated with each virus; general symptoms induced upon comovirus infection include mottling, stunting, and systemic mosaic (Stace-Smith, 1981). All comoviruses induce characteristic cytopathological effects in the cytoplasm of infected cells (Francki et al., 1985), and isometric particles (ca. 30nm in diameter) are produced. The classification of most members of the comoviruses is based only on the properties of the virus particles (particle composition, morphology and serological composition).

1.2 Cowpea mosaic virus.

1.2(A) Biological properties.

Cowpea mosaic virus (CPMV) is one of the most extensively studied plant viruses. Since the identification of CPMV (Chant, 1959) extensive investigations, using the most up-to-date technologies, have greatly increased our understanding of the virus. Cowpea mosaic virus is an economically important pathogen of Vigna unguiculata (L.), the common cowpea. These plants are grown throughout the tropics and subtropics and provide food for millions of people and feed for a vast number of livestock (Agrawal, 1964).

The study of cowpea mosaic virus has been facilitated by the availability of good systemic and local lesion host plants. In Vigna unguiculata (L.) the virus multiplies to high titre and may be extracted in gram amounts. In hosts such as Phaseolus vulgaris var. "Pinto" and Vigna unguiculata var. "Early Red" local lesions are produced, allowing virus assay and genetic purification. Cowpea mesophyll protoplasts may also be infected with CPMV (Hibi et al., 1975, Beier and Bruening, 1976). After inoculation, up to 80% of protoplasts become infected, infection occurring synchronously thus allowing studies on CPMV replication and the detection of viral encoded non-structural proteins (Rottier et al., 1979, 1980a, 1980b).

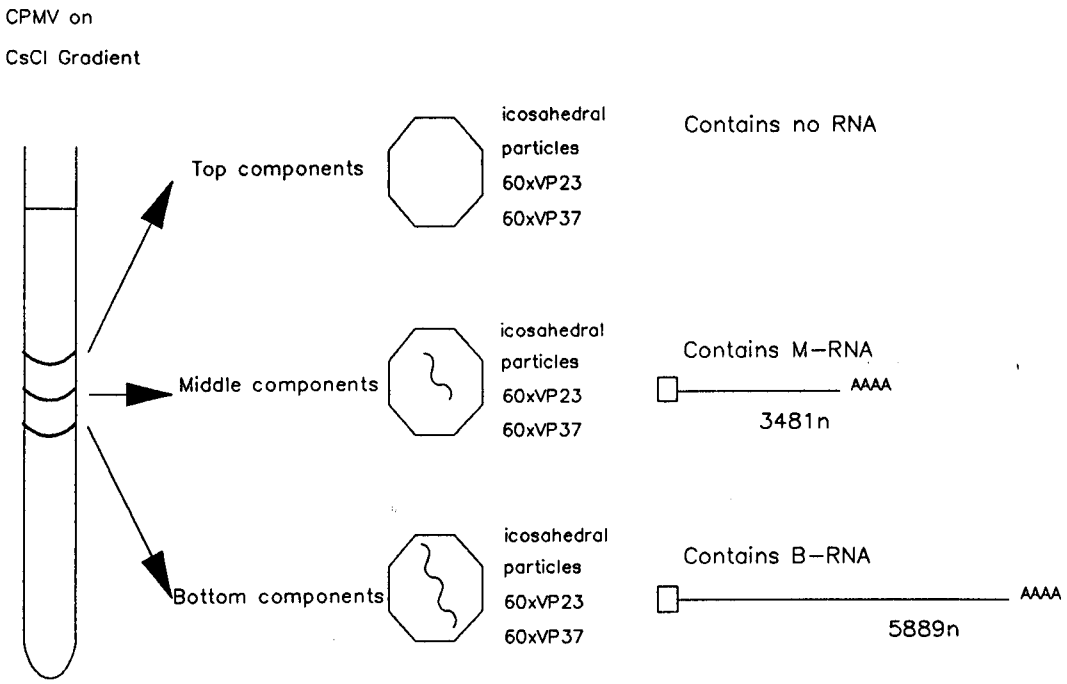


FIGURE 1.1

Figure to illustrate that a preparation of cowpea mosaic virus consists of three components.

Particles of CPMV are built from sixty copies of each of the two coat proteins, VP37 and VP23. The capsid structure has been determined to 3.5Å resolution and shows a pseudo T=3 icosohedral symmetry (Stauffacher et al., 1987). Genuinely T=3 plant viruses,such as tomato bushy stunt virus and southern bean mosaic virus, are composed of 180 identical coat protein subunits, each subunit occupying one of three different positions within the virion. The two coat proteins of CPMV consist of three distinct β-barrel domains, two being derived from the VP37 and one from VP23; each of the β-barrel domains occupies one of the three different positions within the virion resulting in pseudo T=3 icosahedral symmetry.

Cowpea mosaic virus, like all comoviruses, can be separated into three types of particle by bouyant density centrifugation. The particles are referred to as bottom (B), middle (M) and top (T) components respectively and have an identical protein composition. The three components differ only in their nucleic acid content, the bipartite RNA genome being split between the B and M components with the T components being devoid of RNA, (Bruening, 1978; Francki et al, 1985). Figure 1.1 illustrates this tri-component nature of CPMV.

The middle and bottom components are both required to give an infection in susceptible plants. In contrast, the bottom component may replicate independently in protoplasts and in the initially infected cells of inoculated leaves (Goldbach et al., 1980; Rezelman et al., 1982). However, no virus particles are produced in protoplasts which have been inoculated with B RNA alone. Further, compared with a mixture of M RNA and B RNA, inoculation with B RNA alone results in reduced RNA accumulation. This, and other data on the stability of M RNA in protoplasts, suggests that the genomic RNAs are unstable in vivo when not protected by coat protein (De Varennes and Maule, 1985).

1.2(B) RNA structure.

The single-stranded messenger-sense RNA genome of CPMV is bipartite, the genome segments separately encapsidated in bottom and middle components being known as B RNA and M RNA respectively. The terminal structures of the RNAs have been characterised. Both M RNA and B RNA have a 3' poly(A) tail and a 5' "viral protein, genome bound" or "VPg" (El manna and Bruening, 1973; Stanley et al., 1978; Daubert et al., 1978). The VPg, 28 amino acids long and encoded by CPMV B RNA, is covalently linked to the 5' terminal uridine of both genomic RNAs by a phosphodiester bond with its amino-terminal serine (Stanley et al., 1980, Zabel et al., 1984, Wellink et al., 1986, Jaegle et al., 1987).

The poly(A) tails of M RNA and B RNA are thought to stabilise the RNA (Huez et al., 1983), although the length of the poly(A) tail can be significantly reduced from the average of 113 nucleotides without eliminating infectivity (Steele and Frist, 1978; Eggen et al., 1989). It is not clear how the poly(A) tails arise on the RNAs, only the B RNA having the plant polyadenylation signal A/U AAUAA in its 3' non-coding region (Lomonossoff et al., 1985, Heidecker and Messing, 1986). In addition, replicative form RNAs isolated from CPMV infected plants have poly(U) sequences at their 5' ends implying that the poly(A) tails are at least partially synthesised on a template (Lomonossoff et al., 1985).

The nucleotide sequence of the CPMV RNA reveals that each genome segment possesses a single large open reading frame (Van Wezenbeek et al., 1983, Lomonossoff and Shanks, 1983). Excluding the poly(A) tail, M RNA has a sequence of 3481 nucleotides. The long open reading frame starts at position 161 and continues until a UAA stop codon at nucleotide 3299 and codes for a protein of predicted molecular weight 116 085. The B RNA is 5889 nucleotides long (excluding the poly(A) tail) and contains an open reading frame which starts at

position 207 and continues to an UAG terminator at position 5805. This reading frame can code for a polypeptide of molecular weight 207 760.

1.2(C) Expression of the bottom component RNA.

Consistent with the primary structure of CPMV RNA, B RNA may be translated in the wheat germ system to produce a protein of approximately 200 kilodaltons (Davies et al.,1977). In principle, translation of CPMV B RNA in reticulocyte lysates and in wheat germ extracts should yield the same polypeptides. This is, however, not the case, the B RNA being translated in reticulocyte lysates to produce a variety of smaller products (see below; Pelham 1979; Franssen et al., 1984a). The protein of approximately 200 kilodaltons is synthesised by B RNA in reticulocyte lysates only when proteolysis is inhibited (Pelham 1979). These translation studies in reticulocyte lysates provided the first evidence that the expression of CPMV B RNA involves proteolytic cleavage of a primary translation product. Cleavage of the B RNA-encoded primary translation product does not occur in wheat germ extracts since a natural inhibitor of proteolysis is present (Shih et al., 1987).

The model of B RNA translation and processing shown in Figure 1.2 is based on in vitro and in vivo data. (The proteins encoded by the M RNA and B RNA are named according to their apparent molecular weights as estimated by polyacrylamide gel electrophoresis. Thus, the protein of apparent molecular weight 200kDa is known as the 200K protein, despite its size as calculated from the nucleotide sequence being 208kDa. All CPMV-encoded proteins are likewise named according to their apparent molecular weights.).

The expression of B RNA in vitro has been mainly studied in reticulocyte lysates. The processing observed in such lysates has been demonstrated to be a faithful representation of the in vivo situation (Goldbach et al., 1981; Franssen

FIGURE 1.2.

Model for the expression of CPMV B RNA.

The B RNA is represented by a line. The open reading frame on B RNA starts at the nucleotide position 207 and continues until position 5805.

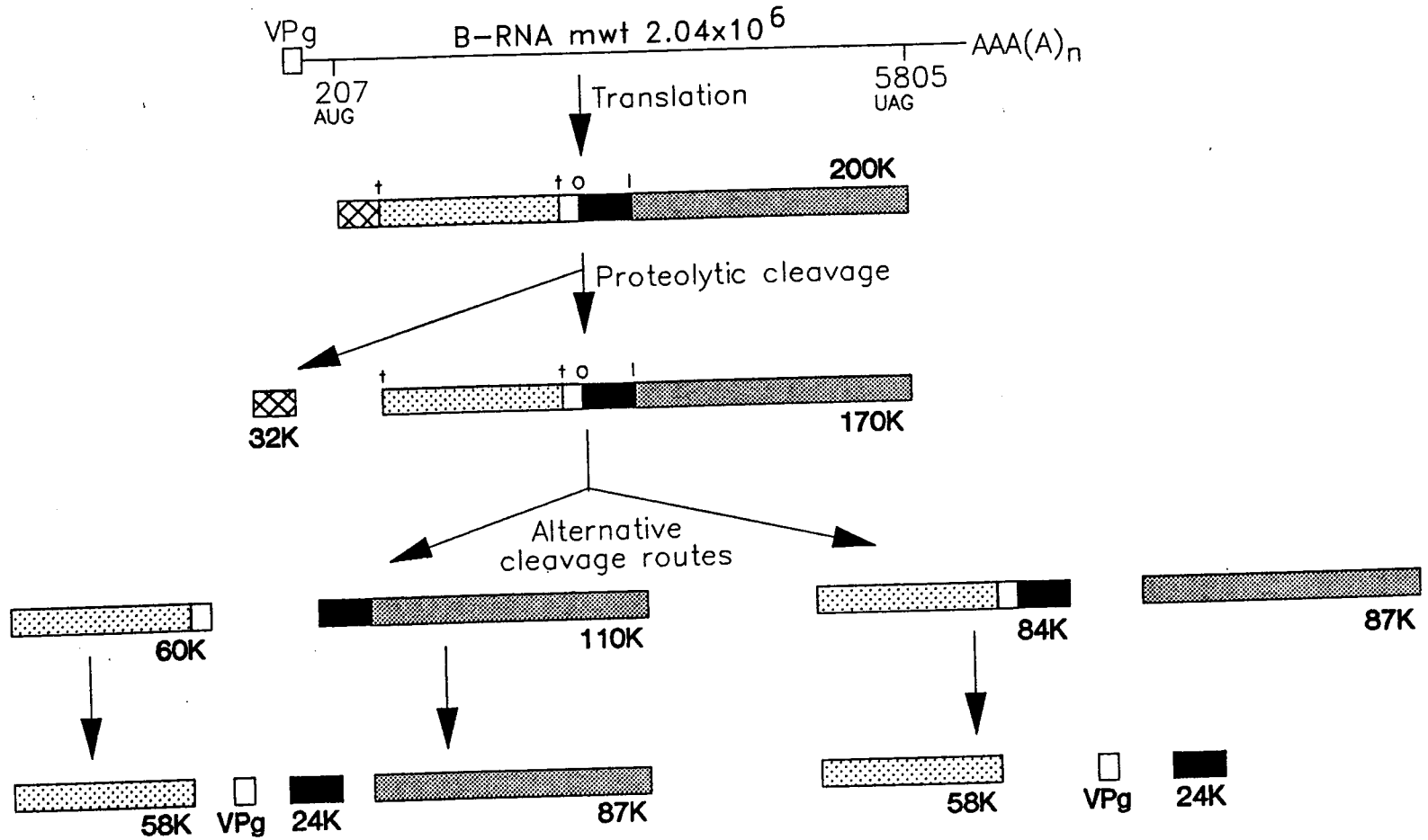
The B RNA-encoded proteins are represented by rectangles.
These proteins are named according to their apparent molecular weights (K).
The cleavage sites used during proteolytic processing are:

† glutamine-serine.

o glutamine methionine.

| glutamine-glycine.

Model For The Expression Of B-RNA



et al., 1984a). For in vivo studies both plants and protoplasts have been used (Rottier et al., 1980a; 1980b; Rezelman et al., 1980; Wellink et al., 1987a; 1987b). The 200K primary translation product found in in vitro translation experiments has never been detected in infected plants or protoplasts presumably because it is processed rapidly (Peng and Shih, 1984). The 200K protein is the immediate precursor to the 32K and 170K proteins. Subsequent processing at the three cleavage sites within the 170K may occur in a different order but lead eventually to the production of proteins of 87K, 58K, 24K and the VPg. All five final cleavage products of the 200K protein have been identified in CPMV infected protoplasts although VPg which is not linked to RNA or part of a precursor has never been detected in vivo, (Rezelman et al., 1980; Goldbach et al., 1982; Wellink et al., 1986). In addition to the 60K precursor of the VPg, the other processing intermediates, the 110K and 84K, have been detected in protoplasts infected with B RNA (Rezelman et al., 1980).

The relationships between the proteins encoded by B RNA and their order in the genome was initially defined by proteolytic peptide mapping and by screening for the presence of VPg sequences in the various cleavage intermediates with anti-VPg serum (Rezelman et al., 1980; Zabel et al., 1982; Goldbach et al., 1982; Franssen et al., 1984a). Goldbach and Rezelman (1983) postulated the cleavage map of the 200K protein to be NH_2-32K protein-58K protein-VPg-24K protein-87K protein-COOH and this has been confirmed by partial amino acid sequencing of isolated B RNA encoded proteins and alignment of these sequences with the open reading frame in B RNA (Wellink et al., 1986). This work also revealed that three types of cleavage site are used to process the 200K protein, namely glutamine-serine (2x), glutamine-methionine and glutamine-glycine amino acid pairs (see Figure 1.2).

Functions have been assigned to several of the B RNA encoded proteins. Since

B RNA may replicate independently in protoplasts, all virus-encoded factors necessary for replication must be present on this genome segment. The 110K protein is present in CPMV replication complexes purified from infected leaves (Dorssers et al., 1984) and has therefore been tentatively designated the CPMV replicase. The 87K protein, which makes up the carboxy terminus of the 110K protein, has been designated the putative core polymerase by virtue of homologies with poliovirus polymerase 3D (Franssen et al., 1984b).

The similarities which exist between comoviruses and picornaviruses have also helped in the assignment of functions to the 24K and 58K proteins. The 24K protein is homologous to the picornavirus protease 3C and is now believed to be responsible for all cleavages of the CPMV polyproteins (Verver et al., 1987; Vos et al., 1988a). Originally, the 32K protein was designated the protease responsible for cleavage of the M RNA-encoded 95K and 105K proteins (Franssen et al., 1984c). However, more recent evidence, obtained using cDNA clones which are hybrids between M and B RNA, suggests that the 32K protein is a cofactor which assists the 24K protease in the cleavage of the 60K coat protein precursor from the M RNA-encoded primary translation products rather than a protease itself (Vos et al., 1988a).

The function of the B RNA-encoded 58K protein is uncertain. Both this protein and its 60K precursor are membrane bound. Since replication is known to occur on membraneous vesicles in infected cells these proteins may be required to anchor all proteins necessary for replication into position. The 58K protein is homologous to the poliovirus 2C protein which is also membrane bound and thought to be associated with RNA replication (Franssen et al., 1984b; Tershak, 1984).

The function of the VPg is not certain. The VPg is not essential for infectivity (Stanley et al., 1978; Vos et al., 1988b) and is not unlinked from the

RNA during in vitro translation (De Varennes et al., 1986). A role of VPg, or the generation of VPg, in viral RNA synthesis has been proposed (Eggen and Van Kammen, 1988). A VPg is found at the 5' end of both negative and positive RNA strands in the replicative forms isolated from CPMV-infected leaves (Lomonossoff et al., 1985) so may have a role in the early stages of CPMV RNA replication. There is stronger evidence that the VPg of poliovirus is involved in the initiation of RNA replication. It has been shown that a crude membrane fraction from poliovirus-infected HeLa cells is capable of synthesising in vitro the uridylated proteins VPg-pU and VPg-pU-pU, which under conditions of RNA synthesis are further elongated into much longer stretches of 5' terminal poliovirus RNA (Takeda et al., 1986). This data suggests that VPg-pU might function as a primer for viral RNA synthesis. For CPMV there is no equivalent experimental information on the role of VPg during the early stages of replication since no in vitro system for CPMV RNA replication has been developed.

1.2(D) Expression of the middle component RNA.

When M RNA is translated in reticulocyte lysates and wheat germ extracts, two over-lapping polypeptides of approximately 100kDa known as the 105K and 95K proteins are synthesised (Pelham and Jackson, 1976; Davies et al., 1977; Pelham, 1979). The 105K and 95K proteins are carboxy-coterminal, being produced following initiation of translation at two independent AUG codons on the M RNA (Vos et al., 1984). Before the work presented in this thesis, there was little evidence to indicate which of the AUG codons at the 5' end of the M RNA were used as initiators of protein synthesis. Inspection of the of the nucleotide sequence of M RNA reveals that the first AUG (at position 115) is shortly followed by an in-phase terminator (van Wezenbeek et al., 1983). The long open reading frame which starts at position 161 could encode a protein of

molecular weight 116 085, a plausible candidate for the 105K protein (Van Wezenbeek et al., 1983). After the AUG at position 161 the next AUG codons in the same reading frame are at positions 512, 524, 632, 674 and 716; initiation at these AUGs predicted to give proteins of molecular weights 102 212, 101 752, 97 730, 96 003 and 94 614 respectively. Initiation at any one of these AUG codons could lead to the synthesis of a "95K" protein.

At the commencement of this thesis there was no direct evidence that the dual initiation of translation observed in vitro also operated in vivo. The primary translation products (or their N terminal cleavage products) had never been detected in CPMV infected plants or protoplasts. However, it is a striking fact that the M RNAs of all comoviruses studied so far (cowpea severe mosaic virus, bean pod mottle virus, red clover mottle virus and squash mosaic virus), all produce two proteins upon in vitro translation (Beier et al., 1981; Heibert and Purcifull, 1981; Goldbach and Krijt, 1982; Gabriel et al., 1982), supporting the idea of that the dual initiation of translation is important to the replication cycle of comoviruses.

The genes of M RNA, like those of B RNA, are expressed by proteolytic processing of precursor proteins. The model for M RNA translation and processing is presented in figure 1.3. Initially the model was based entirely on in vitro translation experiments but is now supported by some in vivo data.

The 105K and 95K overlapping proteins translated from M RNA in reticulocyte lysates are cleavaged to give the 48K, 58K and 60K proteins, the cleavages being dependent on the presence of in vitro or in vivo generated B RNA-encoded 24K and 32K proteins (Pelham, 1979; Franssen et al., 1982; Vos et al., 1988a). The 60K protein is the precursor of the coat proteins (Franssen et al., 1982) but its cleavage to VP37 and VP23 has not been demonstrated in vitro.

Until the recent detection of the 60K and 48K proteins in protoplasts (Wellink

FIGURE 1.3.

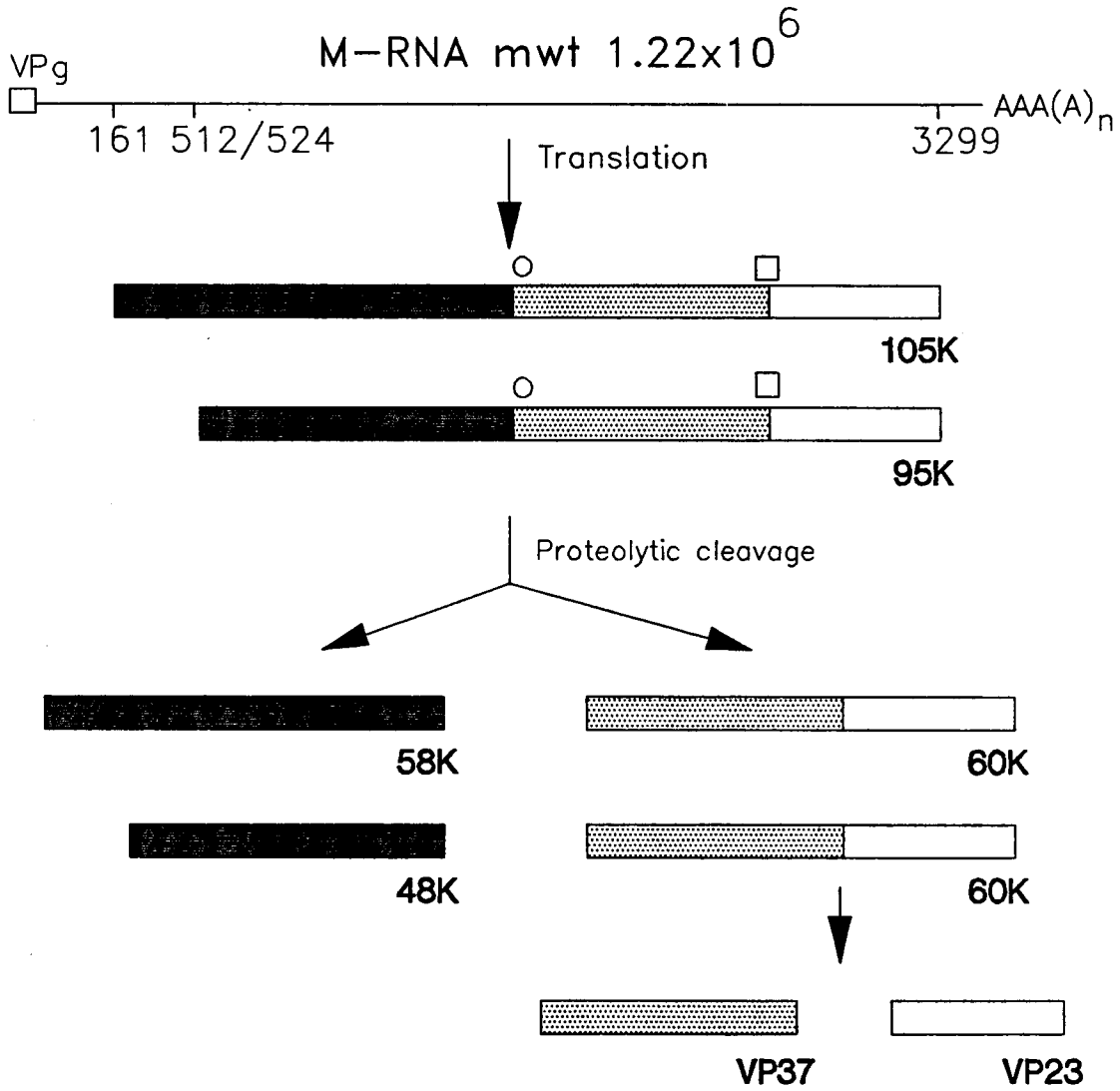
Model for the expression of CPMV M RNA.

The M RNA is represented by a line. The numbers below the M RNA indicate the positions of the first three AUG codons that are in-frame with the termination codon at position 3299.

The M RNA-encoded proteins are represented by rectangles.
These proteins are named according to their apparent molecular weights (K).
The cleavage sites used during proteolytic processing are:

- o glutamine-methionine.
- ◻ glutamine-glycine.

Model for the expression of M-RNA



et al., 1987a), the only M RNA encoded proteins found in vivo were the two coat proteins (Goldbach et al., 1980). The 60K precursor to the coat proteins is detected only in protoplasts which have been incubated in the presence of zinc chloride, presumably because the zinc ions inhibit the proteolytic cleavage of the 60K protein (Wellink et al., 1987a). These workers also detected the 48K protein in infected cowpea leaves and protoplasts using sera raised against synthetic oligopeptides, the sequence of the oligopeptides corresponding to the common C-terminal nine or thirty amino acids of the 48K and 58K proteins. The occurrence of the 60K and 48K proteins in infected cells suggests that the M RNA is expressed in vivo by polyprotein processing as observed in vitro.

The proteolytic cleavage sites on the M RNA are a glutamine-methionine pair and a glutamine-glycine pair (see Figure 1.3) and have been determined by mapping the N-terminal protein sequences of VP37 and VP23 on to the nucleotide sequence of M RNA (Van Wezenbeek et al., 1984).

Functions have been assigned to several of the M RNA encoded proteins. The two coat proteins encapsidate, and thus protect, the genomic RNAs. Little is known about the mechanisms of capsid assembly and RNA encapsidation though the existence of capsids which are devoid of genomic RNA (top components) suggests that protein-RNA interactions are not essential for assembly. The possible involvement of other proteins, besides the capsid proteins, in these processes has not been investigated.

The 58/48K overlapping proteins are thought to have a function in the cell to cell spread of CPMV within an infected plant. Indirect evidence for this function is provided by the observation that B RNA replication is restricted to the initially infected leaf cells (Rezelman et al., 1982). This implies that an M RNA-encoded function is required for spread of the RNA. It is posssible that

encapsidation is a prerequisit for transport, and/or the 58/48K proteins may have a function in virus movement throughout the plant.

1.2(E) Replication of the CPMV genome.

Attempts to define the mode of replication of the CPMV genome have been hampered by the inability to isolate a template-dependent CPMV polymerase from infected plant material. The CPMV replication complexes which have been isolated are only capable of elongating RNA molecules which initiated in vivo (Dorssers et al., 1984). Furthermore, the feasibility of studies based on infected plants is adversely affected by the presence of a host-encoded RNA-dependent RNA-polymerase (RdRp). In CPMV-infected plants the activity of this host enzyme is enhanced to such an extent that the activity of the polymerase involved in CPMV replication accounts for less than 5% of total RdRp activity (Dorssers et al., 1983). The biological function of this host encoded enzyme (Mr.=130kDa) still remains to be determined. It has been shown to transcribe plant and viral RNAs into short RNA molecules of negative polarity but is not thought to be involved in the replication of the CPMV genome (Van der Meer et al., 1984).

The complications encountered when using infected plants as a source of CPMV RNA polymerase have led to attempts to synthesise the CPMV replicative machinery in a prokaryotic expression system (Richards et al., 1989). The B RNA encoded 110K protein and the 87K protein were expressed in an E.coli system which had previously been used successfully for the expression of the polioviral polymerase. Although the proteins were synthesised no polymerase activity was detected. One explanation for this inactivity is the requirement of a host factor in CPMV replication; indeed, replication complexes purified from CPMV-infected plants do contain host proteins and CPMV replication can be inhibited during the

early stages of infection by the addition of actinomycin D, a potent inhibitor of DNA-dependent RNA synthesis (Dorssers et al., 1984; Rottier et al., 1979; De Varennes et al., 1985).

Cells infected with CPMV develop vesicular membranes of characteristic cytopathic structure. These structures are thought to be associated with replication since, in addition to an abundance of B RNA encoded non-structural proteins (Wellink et al., 1988), the cytopathic structures also contain double stranded genomic RNAs (De Zoeten el al., 1974).

In a model for CPMV replication recently proposed by Eggen and Van Kammen (1988), a replication complex forms between the membrane-bound 60K precursor to VPg, the 110K protein (which contains the 87K putative core polymerase and the 24K protease), the RNA template and a possible host factor. It is proposed that RNA replication is closely linked to proteolytic processing, the VPg being released, possibly being used as a primer and then transcription beginning. Since each replication complex contains only one VPg, the replicative machinery is used only once. This model is consistent with the general observations made with regard to CPMV replication but, as yet, there is no experimental evidence for its operation.

1.3 Use of mutants to investigate gene function in viruses.

1.3(A) Traditional Genetics.

Mutants have been extensively applied to the genetic analysis of viruses. The ultimate aim of genetic studies is to gain a detailed understanding of the structure and function of the viral genome and each of the viral gene products. Traditionally, studies of viral genetics centred on the collection and subsequent genetic and physiological characterisation of virus mutants.

Most mutants isolated during studies of viruses have been derived from

mutagen-treated populations of the wild-type. Chemical and physical mutagens have been used to increase the frequency of mutation in plant viral genomes. Chemicals such as nitrous acid, hydroxylamine, bisulfite and alkylating agents, which induce specific chemical modifications in nucleic acids, have been more widely used than chemicals such as 5-fluorouracil which is an analog of uracil and mutagenic when incorporated into genomic RNA in vivo. Of the physical mutagens, ultraviolet light has been used occasionally.

In addition to induced mutants of plant viruses, spontaneous viral mutants have also been isolated and studied. Spontaneous mutations arise in the genomes of viruses as a result of errors during replication. Viruses with RNA genomes show higher mutation frequencies (10^{-3} to 10^{-4} per incorporated nucleotide) when compared with viruses that replicate from a DNA template (10^{-8} to 10^{-11} errors per incorporated nucleotide). These differences in rates of mutation occur because replicases that synthesis progeny from an RNA template lack the proofreading activities that assure fidelity of replication from a DNA template (Holland et al., 1982). Spontaneous mutation provides the variation essential for viral evolution and also provides a source of mutants for study by the geneticist.

Initially, viral mutants are isolated from a mixed population by selection for a desired phenotype. Mutants of plant viruses have been selected on the basis of characters such as aberrant symptom formation, altered host range and temperature sensitivity. Subsequently, the virus mutants are genetically purified to produce a virus population ideally originating from a single infectious unit. The use of plaque assays allows the production of clonal strains of animal viruses, each focus of infection on a tissue culture monolayer resulting from a single infectious unit. Unfortunately, there are no analagous tissue culture systems for use with plant viruses so mutants are isolated by propagation in intact host plants. Inoculation of local lesion hosts with plant viruses does produce discrete

foci of infection, but evidence suggests that local lesions do not necessarily contain clonally derived virus. A theoretical consideration of dilution curve data (ie. lesions produced against dilution of virus inoculated) indicates that a single infectious unit is capable of producing a lesion (Furumoto and Mickey, 1967). However, Garcia-Arenal et al., (1984) found that genetically homogeneous virus was not obtained by local lesion passage at high dilution. In addition to genomes with slight alterations arising by natural mutation, the predominant genotype was contaminated with variants having 30-40% sequence divergence. This second class represent distantly related strains which are passaged as contaminants of the original inoculum.

The inability to produce clonally derived plant virus by local-lesion passage stems from the inefficiency of the infection process. It has been estimated that 10^6-10^8 particles of CPMV must be mechanically inoculated for each lesion produced (Van Kammen, 1968). The inefficiency of infection is a result of the inoculation process; virus enters the leaf cells only at wounds produced during the mechanical inoculation process. Since only a proportion of the cells will be infectible and the lifetime of their wounds short, a large number of virus particles is required to ensure successful infection of the susceptible sites. It is also thought that a high proportion of the inoculated virus will not even come into contact with infectible sites owing to its uneven distributed over the leaf and/or binding to inactive sites on the leaf surface (Matthews, 1981).

The single-lesion passage is the only practical biological procedure available to genetically purify plant virus preparations yet its efficacy is not guaranteed. In addition, although spontaneous mutation provides a source of mutants for genetic analysis, the same process may also be viewed as disadvantageous, resulting in difficulties maintaining a homogenous virus population. The genetic purification of plant viruses therefore results in a heterogeneous mix in which one genotype

predominates.

Despite these difficulties in genetic purification of plant viruses, mutants have been successfully isolated and studied. Mutants of viruses with a divided genome have been used to assign specific functions to a particular RNA species. A property is assigned to a particular genome segment or segments by mixing wild-type and mutant components in a variety of combinations or by phenotype rescue after the addition of individual wild-type components. Many studies have used mutants to map functions to genome segments in this way but in relatively few cases has a detailed biochemical characterisation been attempted. This is probably because at the time of most of the studies, relatively little was known about the biochemistry of the wild-type virus infection.

In the case of CPMV, both spontaneous and induced mutants have been isolated and studied. To induce mutations the chemical agent nitrous acid has been favoured, the one exception being mutant Bil induced with bisulfite (Siler et al., 1976). After contact with the mutagen, a variety of methods have been used for mutant selection, the most popular being aberrant lesion formation (Wood, 1972; de Jager, 1976; de Jager et al., 1977; de Jager and Breekland, 1979; Evans 1985a; 1985b). However, the ability of the virus to be passaged on a resistant variety of cowpea (Siler et al., 1976) and screening for an altered electrophoretic profile of virions (Gopo and Frist, 1977), have also been used.

Some of the first mutants to be isolated had functional defects associated with the coat proteins. The extent of top component production (postulated to be a result of excess capsid protein synthesis) was mapped on to the M component of a spontaneously produced mutant, (Bruening, 1969). Chemically-induced mutants with aberrant coat proteins were also isolated by Wood (1972), Gopo and Frist (1977) and Siler et al. (1976). The mutant Bil (Siler et al., 1976) and the mutant G3 (Gopo and Frist, 1977) both have altered electrophoretic characteristics of

their virus particles. Both wild-type and Bi1 have two electrophoretic forms but the normal conversion between the two forms occurs at a faster rate in the Bi1 mutant. Mutant G3 differed from wild type in demonstrating an additional electrophoretic form. By investigating the electrophoretic characteristics of wild-type/mutant heterologous mixes, the mutation in Bi1 was assigned to the B RNA and that in G3 to the M RNA. The conversion between the wild-type electrophoretic forms is a result of removal of a labile peptide at the C-terminus of VP23 (Niblett and Semancik, 1969; Kridl and Bruening, 1983) so the mutation in G3 may be mapped onto this coat protein. With regard to Bi1, there is not sufficient information to identify a possible aberrant protein although the authors suggest that an altered B RNA-encoded protease might be responsible for the different rates of cleavage of VP23.

De Jager and his co-workers have made an extensive genetic study of CPMV mutants, isolating both spontaneous (de Jager and Wesseling, 1981) and nitrous acid-induced mutants (de Jager, 1976; de Jager et al., 1977; de Jager and Breekland, 1979). In addition to illustrating that symptom expression determinants may be mapped on to either genome segment, the work revealed problems inherent in techniques used for mapping analysis. The addition of wild-type M or B component to a mutant virus to rescue the wild-type phenotype was not always found to give an accurate reflection of the location of the mutation (de Jager et al., 1977; de Jager and McLean, 1979). The most accurate method for mapping is in vitro recombination where separated M or B components from the mutant are mixed with wild-type B or M component and the phenotypes of the progeny analysed. However, this test can not be applied to mutants that do not grow well, since insufficient material is available for component purification.

A nitrous acid induced mutant (7A-4) isolated by Evans (1985a) was originally selected on the basis of aberrant lesion formation on Phaseolus vulgaris and found

subsequently to be unable to grow in cowpea (Vigna unguiculata) varieties "Early Red" or "Blackeye Early Ramshorn". The genetic defect was mapped on to the B RNA but further characterisation was limited by the inability to propagate the mutant efficiently.

The most useful induced mutants are those which are conditionally lethal such as the temperature sensitive mutants. These "ts" mutants have an approximately wild-type phenotype at a certain temperature (the permissive temperature) and an abberant phenotype at a higher temperature (the restrictive temperature). Each ts mutant has a "built in control" in that an essentially wild-type phenotype is observed under permissive conditions, although ts mutants rarely propagate as effectively as wild-type. When ts mutants are characterised to reveal a function that is mutant, a virus-specified activity is therefore revealed. The time in the virus life-cycle at which that activity is required can then be determined by temperature-shift experiments after synchronous infection of protoplasts. Temperature shift experiments have been used during the analysis of UV-induced ts mutants of alfalfa mosaic virus, the phenotypic lesion affecting early functions of the replication cycle (Huisman et al., 1985).

The great advantage of conditionally lethal mutants is that, potentially, any region of the genome may be mutated. It is in theory possible to isolate ts mutants that have alterations at the restrictive temperature in essential functions that can not be genetically dissected in any other way. The experimental objective is to collect and study a series of ts mutants of a particular virus, determine the nature of each ts lesion and and to assign each lesion to a particular region of the genome. Using this approach, it should be possible to assign all the essential functions to particular virus proteins or cis-acting regions of the genomic nucleic acid.

Three temperature sensitive mutants of CPMV have been studied. All were

induced with nitrous acid, initially selected on the basis of aberrant lesion formation and then shown to be temperature sensitive. The mutant N168 has a ts defect on M RNA which affects virus accumulation (de Jager et al., 1977; de Jager and McLean, 1979). In an attempt to define the physiological lesion, a putative replicase was isolated from N168-infected cowpeas and analysed for a ts effect in RNA synthesis. No difference was observed between RNA synthesis activity isolated from wild-type or N168 inoculated plants. The ts character in virus accumulation was then postulated to be due to the absence of the synthesis of replicase activity at the restrictive temperature and not thermolability of the replicase complex. However, with insight into the genomic map of CPMV and the mapping of replicase functions onto the B RNA (Goldbach, et al., 1980) this theory is no longer tenable. Since naked RNA is not stable in vivo (de Varennes and Maule, 1985) it is probable that N168 is actually ts with respect to encapsidation rather than replication, probably as a result of a mutation(s) in the coat proteins which are encoded by M RNA.

Two mutants isolated by Evans (1985b) are also ts in virus accumulation, the defect mapping on to the B RNA of mutant 8-14 and being associated with both RNAs of mutant 8-10. At the restrictive temperature, small amounts of RNA were detected but no virus coat protein or infectious virus produced for both mutants. Further characterisation of mutant 8-14 is included in this thesis.

1.3(B) Reverse Genetics.

Mutants produced by traditional methods were particularly useful when little was known about the protein expression and multiplication cycle of plant viruses. The ability to isolate a mutation in any region of the genome being a valuable and relevant attribute of the conditionally lethal mutant. More recently, the nucleotide sequence of many plant virus genomes has been determined and thus

the primary structures of putative virus encoded proteins revealed. In addition, our understanding of the biochemistry of virus infections is increasing. As a result, the potential now exists to make informed guesses about the expression and replication cycle of certain viruses. In order to profit from such guesses, a means of engineering specific mutations into the viral genome is required. For the majority of plant viruses whose expression does not involve DNA intermediates, the standard techniques of genetic engineering initially could not be readily applied. The ability to produce "infectious clones" (see below) of viral RNA genomes has therefore considerably increased the potential for molecular studies of RNA viruses. It is possible to introduce desired mutations at any site of an RNA sequence by working at the cDNA level and using standard recombinant DNA techniques.

Full length infectious cDNA clones were first demonstrated for bacteriophage QB (Taniguchi et al.,1978) and poliovirus (Racaniello and Baltimore, 1981). These cDNA clones were shown to be directly infectious but only at a low level (the specfic activity of poliovirus cDNA is at best two to three orders of magnitude lower than that of virion RNA, Semler et al., 1984). The mechanism by which the cDNA clones directly initiate an infection is not known. Presumably it involves transcription of the plasmid DNA in host cell nuclei and possible processing of the resulting RNA to give biologically active molecules which can be translated and replicated in the cytoplasm. It is therefore not surprising that the efficiency of infection by cDNA clones is low. To date, the only plant virus cDNA found to be directly infectious is a RNA 3 clone of alfalfa mosaic virus (ALMV). A low frequency of infection was detected from the RNA 3 circular DNA copy, three out of twenty tobacco plants becoming infected after co-inoculation with ALMV RNAs 1, 2 and 4, (Dore and Pink, 1988).

The biological activity of other full-length cDNA clones of plant viruses is

expressed only after in vitro transcription of the cDNA, this process generating RNA copies which resemble the virion RNA. Efficient in vitro transcription systems have been described based either on E.coli RNA polymerase or on RNA polymerases of bacteriophage T7, T3 or SP6. Vectors based on the T7 polymerase have been favoured as this enzyme is highly selective for its own promoters (Chamberlin et al. 1970), is capable of generating complete transcripts of very long DNAs from a variety of sources and is at least ten times more active in vitro than E.coli polymerase (Janda et al., 1987). The other bacteriophage polymerases share these advantageous characteristics but to date, only the T7 polymerase gene has been cloned (Davanloo et al., 1984).

A general disadvantage of the phage polymerases is that the promoter consensus sequences extend in to the transcript so transcripts have additional bases at their 5' termini. It has been demonstrated that non-viral extensions at the transcript termini, in particular the 5' end, may adversely affect infectivity (Dawson et al., 1986; van der Werf et al., 1986; Janda et al.,1987; Ziegler-Graff et al., 1988; Heaton et al., 1989). However, it has recently been shown that the promoter consensus sequences may be altered without significantly reducing promoter activity (Janda et al., 1987). Of the nucleotides downstream of the initiation site, two guanosine residues are preferable but initiation at a single guanidine residue is possible.

Infectious T7-generated transcripts with two 5' non-viral guanosine residues have been reported for CPMV (Vos et al., 1988b), beet necrotic yellow vien virus (BNYVV) RNAs 1 and 2 (Quillet et al., 1989), turnip crinkle virus (Heaton et al., 1989) and tobacco rattle virus (Angenent et al., 1989). Infectious transcripts of tobacco vien mottling virus with two extra guanidine residues at their 5' ends can be generated using T3 polymerase. Infectious transcripts of brome mosaic virus (Janda et al., 1987), BNYYV RNAs 3 and 4 (Zeigler-Graff et al., 1988) and

tobacco vein mottling virus (Domier et al., 1989) with a single extra guanosine residue at their 5' ends have been generated using T7 polymerase. The T7-generated transcripts of barley stripe mosaic virus (Petty et al., 1988) and turnip yellow mosaic virus (Weiland and Dreher, 1989) have authentic 5' ends since initiation occurs at the guanosine which is the 5' residue of native genomic RNAs. Biologically active transcripts of alfalfa mosaic virus RNA 4 (Loesch-Fries et al., 1985; Langereis et al., 1986) and tobacco streak virus RNA 4 (Langereis et al., 1986) have been synthesised using SP6 polymerase.

Ahlquist and Janda (1984) developed a transcription vector (pPM1) based on a modified phage λ promoter known as Pm. This in vitro transcription system uses E.coli RNA polymerase and so is not as efficient as those based on the bacteriophage polymerases. However, the advantage of generating transcripts using E.coli RNA polymerase is that sequences downstream from the transcriptional initiation site are dispensable for efficient transcription in vitro, thus generating transcripts with authentic 5' ends. Ahlquist and Janda (1984) cloned the three genomic segments of brome mosaic virus downstream of the Pm promoter. In vitro generated transcripts from these cDNA clones were shown to be infectious (Ahlquist et al., 1984). E.coli RNA polymerase has since been used to generate infectious transcripts from cDNA copies of tobacco mosaic virus (Dawson et al., 1986, Meshi et al., 1986), CPMV M RNA (Holness et al., 1989; this thesis), tobacco rattle virus RNA 2 (Angenent et al., 1989), tobacco rattle virus RNA 1 (Hamilton and Baulcombe, 1989) and turnip crinkle virus (Heaton et al., 1989) which have been cloned downstream of the Pm promoter.

Both E.coli and 'phage-encoded polymerases are capable of incorporating the cap precursor m^7GpppN, so generating capped transcripts. The infectivity of transcripts from plant virus cDNA clones is often enhanced by, or dependent on, the presence of a 5' cap.

Once a cDNA clone has been produced from which infectious transcripts can be synthesised, an enormous variety of specific genetic alterations can be made. The phenotype of the mutant transcripts can then be analysed in vivo, thus allowing information on gene function to be obtained. These directed mutants have both advantages and disadvantages when compared with mutants isolated from virus populations by the traditional methods outlined earlier. Directed mutants will constitute a genetically defined and homogeneous population, circumventing the genetic purification problems associated with traditional mutants. However, of the large number of directed mutants that it is possible to produce, not one is guaranteed to be viable or have an interesting or useful phenotype. The directed mutants also have none of the advantages of the conditionally lethal mutants, ie relative ease of isolation of mutants in essential genes, the "built in" control and the temperature shift experiments. In addition, as there is no selection for the genetic stability of directed mutants, the problems of reversion at the RNA level are still encountered.

The production of directed mutants has significantly facilitated investigations of the functions of some plant viral proteins. As an example, mutants of tobacco mosaic virus (TMV) will be considered. The messenger-sense, single stranded RNA genome of TMV encodes three non-structural proteins (130K, 180K, and 30K proteins) and the coat protein (CP). Transcripts generated in vitro from TMV cDNA clones mutated in each of these genes have been studied in vivo.

The 130K and 180K proteins encoded by TMV are the putative replicase proteins. These proteins have a common N-terminus, the 180K protein being synthesised by read-through over an amber termination codon of the 130K protein gene. Mutations directed at or near the leaky amber termination codon indicated that both the 130K protein and the 180K protein are necessary for normal multiplication in tobacco (Ishikawa et al., 1986). In another investigation of these

genes, base substitutions were directed in to the 130K and 180K proteins (Meshi et al., 1988). Certain amino acids were altered based on sequence information from a resistance-breaking strain of TMV known as Lta1 (Watanabe et al., 1987). In vitro generated mutant transcripts were tested for their ability to overcome the effects of the tomato resistance gene Tm-1. A strong correlation was found between the ability to overcome the resistance and a decrease in local net charge, suggesting the involvement of an electrostatic interaction between the viral 130K and 180K proteins and a putative host resistance factor (Meshi et al., 1988).

A series of TMV mutants with deletions and/or insertions in the CP gene were made by modification of a cDNA clone followed by in vitro transcription (Dawson et al., 1988). All mutants multiplied as free viral RNA and moved from cell to cell in inoculated tobacco leaves. The host response to infection by each of the mutants was observed and correlated with the production (if any) of coat protein-related polypeptides in vivo. The results demonstrated the multifunctional role of the TMV CP gene during infection, including encapsidation, symptom expression and differential elicitation of resistance genes.

Based on information obtained by traditional methods of genetic analysis, directed mutants were produced to show that the TMV 30K protein has a role in movement of the virus from cell to cell, (Meshi et al., 1987). The 30K protein had been tentatively asigned a virus-transport function following the analysis of mutants such as Ls1, a spontaneously-occurring mutant of TMV with a temperature-sensitive defect in virus movement from cell to cell, (Nishiguchi et al., 1978). A difference in the 30K proteins of wild-type and TMV Ls1 had been detected by peptide mapping (Leonard and Zaitlin, 1982), partial sequence analysis of the gene then revealing a single amino acid substitution within the 30K coding region, (Ohno et al., 1983). To confirm that this substitution resulted

in the ts cell to cell movement, an equivalent point mutation was introduced into the 30K gene of an infectious cDNA clone of TMV. Transcripts from the mutant cDNA clone were shown to display a ts phenotype similar to that of TMV Ls1 (Meshi et al., 1987). More genetic alterations were then directed into the 30K gene giving frame shift and deletion mutations. Transcripts bearing these mutations were then tested for replication-competence in protoplasts and infectivity on tobacco plants. The results verified the involvement of the 30K protein in cell to cell spread and so demonstrated that the protein is not essential for replication.

This work on tobacco mosaic virus indicates that directed mutants do have promise for gene function analysis. The number of plant RNA viruses for which infectious full-length cDNA copies are available is growing steadily, so the favouring of directed mutants over traditional mutants for genetic analysis seems inevitable. However, this does not imply that traditional mutants are no longer useful. A recent study on TMV illustrates that the techniques of traditional and reverse genetics may be combined profitably for gene function analysis, (Knorr and Dawson, 1988). During this piece of work, transcripts from a full-length infectious clone of the common strain of TMV were used to infect a systemic host and progeny virions extracted. The virions were treated with nitrous acid to induce a mutant (TMV204-D1) giving a hypersensitive response on Nicotiana sylvestris, a normally a systemic host. A library of cDNA clones of TMV204-D1 was produced and chimeric TMV genomes constructed by exchanging cloned cDNA segments between the local lesion mutant and its wild-type parent. Transcripts from the chimeric cDNA clones were then tested for their response on N. sylvestris. Using this approach a point mutation in the capsid gene was found to be responsible for conferring the ability of TMV to elicit the hypersensitive response in N. sylvestris.

1.4 Scope of the work for this thesis.

For this thesis, it was aimed to study the translation and replication of cowpea mosaic virus using both traditional and reverse genetic techniques. The traditional mutant 8-14, isolated by Evans (1985b), was further characterised. Previous work indicated that, under restrictive conditions, the phenotypic defect in 8-14 resulted in the production of reduced amounts of unencapsidated genomic RNA. Mutant 8-14 was studied in order to define the genetic and physiological lesion(s) responsible for the ts phenotype. The results indicated that a detailed characterisation of 8-14 would be difficult. More emphasis was therefore directed towards the analysis of CPMV by reverse genetic techniques.

To study the translation and replication of CPMV, mutations were directed in to an infectious transcriptional clone of CPMV M RNA. To study the translation of M RNA, the presumptive AUG initiation codons were altered. The translation of mutant transcripts was investigated in reticulocyte lysates and by detecting M RNA-encoded proteins in infected protoplasts. The effect of these mutations on the replication of the transcripts was also studied.

To determine those sequences on M RNA required for it to be replicated by B RNA-encoded proteins, deletion mutants of M RNA were produced. The ability of the deletion mutants to be replicated in cowpea protoplasts was tested.

Chapter 2: Materials and methods.

2.1 Materials.

2.1(A) Enzymes.

Restriction endonucleases were purchased from GIBCO/BRL, Pharmacia or Boehringer Mannheim. Bacterial alkaline phosphatase, T4 polynucleotide kinase, T4 DNA polymerase, calf intestinal phosphatase, Klenow, RNAsin and E.coli RNA polymerase were from Pharmacia. Ribonuclease T1 was from Calbiochem, DNA polymerase I from Anglian Biotech Ltd, T4 ligase from BRL GIBCO, RNAase A was from Boehringer Mannhein, and lysozyme from Sigma. Cellulase R-10 and macerozyme R-10 were from Kinki Yakult Manufacturing Company.

2.1(B) Radiochemicals.

[γ-^{32}P]ATP (5400 Ci/mmol) was purchased from Amersham. All other radiochemicals, namely [α-^{32}P]UTP(800Ci/mmol), [α^{32}P]dCTP (3000Ci/mmol), [5,6-^{3}H]UTP (500Ci/mol) and [^{35}S]Met (1700Ci/mmol), were purchased from New England Nuclear.

2.1(C) Solutions, buffers and reagents.

Deionised water was used throughout and where practicable all solutions were sterilized or prepared from sterile constituents. Most chemicals were of AnalaR grade and supplied by BDH or Sigma. Exceptions to these suppliers are indicated in the text when the specific reagents are mentioned. The following standard solutions, buffers and reagents were used. (All buffers are at 1X unless otherwise indicated):

Calf intestinal phosphatase (CIP) buffer :	50mM Tris-HCl pH 9, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine.
DNA polymerase buffer :	10mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 10mM DTT, 50mM NaCl.
Ligation buffer :	66mM Tris-HCl pH7.5, 10mM $MgCl_2$, 10mM DTT and 10mM rATP for blunt-end ligations or 1mM rATP for sticky-end ligations.
Phosphate buffered saline (PBS) :	4.5mM Na phosphate buffer, pH 7.1, containing 0.85% NaCl.
Phenol (Tris-saturated):	phenol saturated with 100mM Tris-HCl, pH 8.0.
Phenol/chloroform :	1:1 mixture of Tris-saturated phenol with chloroform.
SSC :	1 x SSC is 150mM NaCl, 15mM Na citrate.
Denhart's solution [20X] (Denhart, 1969) :	0.4% ficoll, 0.4% polyvinylpyrollidine, 0.4% bovine serum albumin, 20mM Na_2 EDTA.

Buffers for restriction enzymes:-

Restriction endonuclease digests were performed in the buffers supplied or recommended by the manufacturer.

Gel eletrophoresis buffers:-

MOPS : 20mM 3-[N-Morpholino] propanesulfonic acid [MOPS], 5mM Na Acetate, 1mM Na_2 EDTA pH 7.0.

TBE : 0.089M Tris-base, 0.089M boric acid, 0.002M Na_2 EDTA, pH 8.3.

TEA+SDS : 0.04M Tris-acetate pH 7.0, 0.001M Na_2 EDTA plus 0.1% [w/v] SDS.

Loading buffers for samples before electrophoresis:-

Orange G [5X] : 25mM Na_2 EDTA, pH 7.0, 20% ficoll 400, 0.2% orange G.

Formamide dye mix [4X] : 10ml formamide deionised with 0.5g Amberlite MB1, 0.4ml 500mM Na_2 EDTA, 10mg xylene cyanol, 10mg bromophenol blue.

Protein sample loading buffer [4X] : 0.25M Tris-HCl pH 6.8, 8% SDS [w/v], 40% sucrose [w/v], 20% 2-mercaptoethanol [v/v] and 0.004% bromophenol blue [w/v].

2.1(D) Bacterial strains.

The following derivatives of Escherichia coli K12 were used:-

Strain	Genotype	Reference
JM83	F⁻ ara (lac, proA) rpsL ϕ80dlacZΔM 15	Vieira & Messing (1982).
JM101	(lac, proAB) supE thi /F'traD36 proAB lacI^{q}ZΔM15	Messing (1983)
CJ236	dut, ung, thi, relA; pCJ105 (Cm^r)	Kunkel et al. (1987)
GM242	F⁻ dam-3, recA1, sin-2, thr-1, leuB6, proAZ, his4, metB1, lacY1, galKZ, ara-14, tsx-33, thi-1, deoBG, sup644, rpsL266.	Chater (Pers. comm.)

2.1(E) Media.

The following media were used:-

L-broth (LB): 1% [w/v] Difco bactotryptone, 0.5% [w/v] Difco yeast extract 0.5% [w/v] NaCl, 0.1% [w/v] D-glucose.

L-agar (LA): LB plus 1% agar [w/v].

Hard agar (HA): 1% agar, 1% [w/v] bactotryptone, 0.5% [w/v] yeast extract, 0.05% [w/v] NaCl, 0.01% [w/v] glucose

Soft agar (SA): 0.8% [w/v] bactoagar, 1% [w/v] bactotryptone, 0.8% [w/v] NaCl

2.2 Methods.

2.2(A) Virus, components and RNA.

(A1) Virus isolates and host plants. Two Nigerian isolates of the CPMV strain Sb were used; the first was obtained from Dr. A. van Kammen by Dr G. P. Lomonossoff and used to determine the sequence of CPMV B RNA (Lomonossoff and Shanks, 1983) and the second was obtained from Dr. C. P. de Jager by Dr. D. Evans and used to produce a temperature-sensitive

mutant of CPMV named 8-14 (Evans, 1985b). In this thesis, these isolates will be denoted CPMV.S1 and CPMV.S2 respectively. The mutant 8-14 and three independent revertants of 8-14 were also used.

For virus propagation the systemic host Vigna. unguiculata (L.) Walp. var. "California Blackeye" was used. For virus assay the two local lesion hosts, V. unguiculata var. "Early Red" and Phaseolus vulgaris L. var. "Pinto"; were used. P. vulgaris was also used for genetic purification of virus.

The primary leaves of seedlings which were 7-10 days old were inoculated mechanically. The following four types of inoculum was used, all of which were applied in 10mM sodium phosphate buffer pH 7.0.

i) Sap from systemically infected leaves, (produced by grinding CPMV infected leaves with buffer in a sterile pestle and mortar).

ii) Virus present in a local lesion from P. vulgaris, (the local lesion was isolated and ground with buffer using a serile watch-glass and glass rod).

iii) Purified CPMV virus (1-10ug virus per primary leaf).

iv) CPMV RNA (either RNA from virions or virion B RNA plus transcripts from pPMM2902, a cDNA clone of M RNA).

Virus was propagated in whole plants or detached leaves. Plants were maintained under glasshouse conditions of 18-22^{o}C with daylight supplemented to 16 hours in winter and were grown in individual pots in a 1:1 mixture of John Innes No. 2 compost and peat. Detached leaves were placed on moist filter paper in petri dishes and incubated with continuous illumination at either 20^{o}C or 32^{o}C.

Virus stocks were genetically purified and maintained by local lesion transfer in "Pinto". Dilutions of virus from a "Pinto" lesion were used to inoculate further "Pinto" primary leaves. Leaves which developed 1-20 local lesions 5-7 days post inoculation were stored (short term at 5^{o}C or long term at -20^{o}C) and the local lesions used as sources of further inocula.

(A2) Virus purification, separation of components and RNA extraction. Infected primary leaves of the systemic host were harvested 10-12 days post inoculation and virions purified as described by Klootwijk et al. (1977). Middle and bottom components were separated by centrifugation on 30-60% (w/v) Nycodenz gradients (Nycomed AS, Oslo, Norway) buffered with 10mM sodium phosphate, pH 7.0 (Gugerli, 1984). The gradients were centrifuged at 36,000 rpm for 15 hours at 15°C in a Beckman SW41 rotor. RNA was extracted from virions or the separated components as described by Davies et al. (1978) and resuspended in sterile distilled water (SDW).

(A3) Further purification of CPMV RNAs.

i. **Purification of B RNA:** B RNA for use in protoplast experiments was further purified on 15-30% (w/v) sucrose gradients buffered in NET (100mM NaCl, 10mM Tris-HCl, 1mM Na_2EDTA, pH 7.5) plus 0.1% SDS. The gradients were spun at 32,000 rpm for 12 hours at 15°C in a Beckman SW41 rotor and fractionated into 0.5ml fractions. An aliquot (5ul) of each fraction was taken and the amount of B RNA determined by ethidium bromide fluorescence quantitation after agarose gel electrophoresis (see section 2.2 A4). Fractions containing the leading edge of the B RNA peak were pooled and collected by ethanol precipitation.

ii. **Separation of genomic RNAs:** Genomic RNA segments for fingerprint analysis (section 2.2 B5) were separated on 1% agarose gels buffered with TEA+SDS using TEA+SDS as reservoir buffer. The virion RNA (50ug at 0.25ug/ul) was loaded in the presence of Orange G loading dye and electrophoresis continued for 30-40mins at 75mA. The M RNA and B RNA were then stained with ethidium bromide (1ug/ml), destained for 30min in distilled water (dH_20) and visualised under long wave UV. The separated RNAs were then extracted from the agarose gel by the "Freeze-Squeeze" method essentially as described by Thuring et al. (1975) and their concentration estimated by ethidium bromide fluorescence

quantitation after agarose gel electrophoresis (section 2.2 A4). A yield of 2.5-5ug of each RNA was obtained.

(A4) Quantitation of virus and RNA. The concentration of purified virus and large amounts of RNA (>50ug/ml) was measured spectrophotometrically using a Hitachi U-3200 Spectrophotometer, taking $E_{260}^{0.1\%}=8.1$ for the virus (van Kammen, 1968) and $E_{260}^{0.1\%}=25$ for the viral RNA. All measurements were made using quartz cells of 1cm path length.

The concentration of small amounts of RNA was estimated by **ethidium bromide fluorescence quantitation** after agarose gel electrophoresis (Maniatis et al., 1982). An aliquot of the test RNA (approximately 0.2-2ug) and standards of equivalent RNA of known concentration were analysed by electrophoresis on 1% agarose gels containing formaldehyde (Lehrach et al. 1977). The agarose gels contained 7.5% [v/v] of a 37% [w/v] formaldehyde solution and 1 x MOPS buffer. Samples to be analysed were denatured by mixing with three volumes of a mixture of 67% deionised formamide [v/v], 20% formaldehyde solution -see above-[v/v] and 13% 10 x MOPS [v/v] and heating at 65°C for 5min. Samples were loaded in the presence of Orange G loading buffer and electrophoresis carried out at 75mA for approximately 1 hour with the reservoir buffer consisting of 1X MOPS. When the Orange G dye reached the end of the gels, they were placed in a 1% [w/v] glycine solution for 30min to allow the nucleic acids to renature before staining in ethidium bromide (1ug/ml). The gels were then destained in 1 litre of dH_2O for 1-2 hours or in a covering of dH_20 overnight at 4°C and the nucleic acids detected by illumination of the gel with UV radiation. The gels were photographed using a Polaroid camera and the quantity of RNA in the sample estimated by comparing the fluorescent yield of the sample with that of the standards.

2.2(B) Propagation and analysis of the mutant 8-14.

(B1) Assay for temperature sensitivity. Primary leaves were detached from "Early Red" cowpeas and pairs of opposite leaves were inoculated with a homogenate from a single 8-14 lesion from a "Pinto" leaf. One member of the pair was then incubated at 20°C and the other at 32°C. The number of lesions visible after six days was determined.

(B2) Propagation of 8-14 for virus purification. The virus within a single local lesion on a "Pinto" leaf was used to inoculate five detached primary leaves; a pair from a "Blackeye" plant, a pair from an "Early Red" plant and a "Pinto" leaf. The "Early Red" leaves were used for a temperature sensitivity (ts) assay as described above and the remaining leaves all incubated at 20°C. If the ts assay indicated that the original local lesion contained virus which was temperature sensitive, virus was purified from the systemically infected "Blackeye" leaves as described in 2.2(A2) and the "Pinto" leaf was used as a source of further inoculum.

(B3) Extraction of nucleic acids from inoculated Blackeye leaves. The method of Zelcer et al. (1981) was used. A weighed leaf was ground in a chilled mortar in the presence of liquid nitrogen with 2ml of GPS buffer (0.2M glycine, 0.1M Na_2HPO_4, 0.6M NaCl, pH 9.6), 0.4ml of 10% SDS, 0.004ml 2-mercaptoethanol, 2ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline and 2ml of chloroform-butanol (25:1). The resulting slurry was allowed to melt, transfered to a sterile corex tube and centrifuged at 10K for 5 min to separate the phases, the aqueous phase was removed and precipitated over night with 8ml of ethanol plus 0.3ml of 3M sodium acetate pH 5.0. The precipitate was collected by centrifugation (4 000xg for 20mins, Sorvall RC-5), washed in 70% ethanol, dried under vacuum and resuspended in SDW to a final concentration of nucleic acids from 1mg leaf tissue per 50ul.

(B4) Detection of viral RNA by dot blot hybridisation.

i. **Dot blotting:** To detect viral RNA sequences in the inoculated primary leaves, dot blot hybridisations were performed as described by Maule et al. (1983). Nucleic acids, extracted from leaf tissue as described above, were diluted 1/5 and 1/25 with SDW and 5ul aliquots spotted onto a nitrocellulose filter which had been pre-wetted in 20X SSC. A range of purified virion RNA standards was also applied to the filter. The dried filter was then baked at 80°C for at least two hours. Filters were hybridized with ^{32}P-labelled probes specific for M RNA or B RNA, washed and autoradiographed, (see below).

ii. **Production of M RNA and B RNA specific probes by Nick Translation:-** Replicative-form double-stranded DNA from M13 clones containing either the sequence from nucleotide residues 190-698 of M RNA or the sequence from nucleotide residues 2141-3979 of B RNA were radioactively labelled to high specific activity using E.coli DNA polymerase I and ^{32}P dCTP, as described by Rigby et al.(1977). Unincorporated label was separated from the DNA using the spun-column procedure described by Maniatis el al. (1982).

iii. **Hybridization conditions:** Nitrocellulose filters were pre-hybridized in 3X SSC, 4X Denhart's solution and 50ug/ml denatured calf thymus DNA for 2-3 hours at 65°C. Hybridization was performed in the same buffer on addition of heat-denatured radioactively-labelled DNA, incubation proceding for 18-24 hours at 65°C. The filters were then washed twice in 2X SSC, 0.1% SDS and twice in 0.1X SSC, 0.1X SDS, each wash being performed at 65°C for 30min. Washed filters were wrapped in cling film and autoradiographed at -70°C using intensifying screens (Laskey and Mills, 1977).

(B5) RNA Fingerprinting.

i. **Production of labelled oligonucleotides:** RNA (1 pmole, ie 1.2ug M RNA or 2.2ug B RNA) was digested with T_1 RNase (1unit) in the presence of bacterial

alkaline phosphatase (0.1unit) in 10mM Tris-HCl pH 8.0. The reactions (20ul final volume) were incubated at 37°C for 60min, phenol-chloroform extracted three times, ether extracted three times and the oligonucleotides collected by ethanol precipitation.

The 5' ends of the oligonucleotides were labelled using 10units of T4 polynucleotide kinase and 20uCi [γ-^{32}P-]ATP. The reaction (final volume 20ul) was carried out for 30min at 37°C in 50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 5mM dithiothreitol (DTT), 0.1mM spermidine, 0.1mM EDTA and a total ATP concentration of 3nM ATP. The labelled oligonucleotides were phenol extracted, collected by ethanol precipitation and resuspended in 10ul SDW.

ii. Confirmation of RNA digestion and labelling: A 1ul aliquot of the T_1-digested kinase-labelled RNA was taken, mixed with 3ul dH_2O and 1ul formamide dye mix and boiled for 5min. The denatured oligonucleotides were analysed on a polyacrylamide (19%) bisacrylamide (1%) gel containing 7M urea and TBE. The gel was pre-run at 1200V for 60min using TBE as reservoir buffer. After loading the denatured RNA electrophoresis was continued at 1200V for 90min. The gel was then transfered to a 3MM paper support, wrapped in cling film and autoradiographed at -70°C using intensifying screens (Laskey and Mills, 1977).

iii. 2-D separation of labelled oligonucleotides: Two dimensional polyacrylamide gel electrophoresis was carried out as described by De Wachter and Fiers, (1972). The first dimension consisted of a denaturing 10% acrylamide, 0.325% bisacrylamide gel containing 6M urea and 0.025M citric acid, pH 3.5. Polymerisation was catalysed by the addition of $FeSO_4.7H_20$ (7ug/ml), Ascorbic acid (300ug/ml) and H_2O_2 (850ug/ml). The reservoir buffer was 0.025M citric acid, pH 3.5. The gel was pre-electrophoresed for 1 hour at 250V. The labelled oligonucleotides were loaded in 4M urea (9ul oligonucleotide solution + 9ul 9M urea + 2ul formamide dye mix), and electrophoresis continued at 200V over-night

(approximately 15 hours).

The second dimension consisted of a non-denaturing 19% polyacrylamide 1% bisacrylamide gel at pH 8.3. The reservoir and gel buffer was 1X TBE. The second dimension was prepared by placing the gel strip from the first dimension between the glass plates which were to become the electrophoresis cell, and then pouring the 20% acrylamide/1X TBE gel solution on top. After polymerisation, the gel was electrophoresed, with current running from bottom to top, at 200V over-night. After electrophoresis, the gel was transferred to a 3MM paper support, wrapped in cling film and autoradiographed as described above.

2.2(C) DNA cloning and sequencing.

(C1) Restriction endonuclease digestion. Restriction endonuclease digests were performed in the buffer supplied or recommended by the manufacturer. The proportion of restriction enzyme volume relative to the total digest volume never exceeded 5%. Digests used 1-2u of enzyme per ug DNA and were at 37°C for at least 2 hours. Except for SmaI digests which were performed at 30°C. When small-scale preparations of plasmid DNA were being digested, RNAase A (0.25ug/ul) was included in digest reactions.

(C2) Gel electrophoresis. DNA was mixed with orange G loading dye and electrophoresed at 80mA using flat bed agarose gels in 1X TBE buffer with 1X TBE as reservoir buffer. After electrophoresis, the gels were stained with ethidium bromide, illuminated under UV radiation and photographed. For most purposes, 1% agarose gels were suitable but, when visualising fragments of less than 500bp, 1.5% or 2% gels were used. Size markers used were either kilobase markers (GIBCO/BRL) or λ DNA digested with PstI. For fragments less than 300bp in size, 8% polyacrylamide gels (7.73% polyacrylamide, 0.27% bisacrylamide) in 1X TBE were run using vertical gel tanks as described by Maniatis et al. (1982).

(C3) Isolation of DNA fragments from gels. DNA fragments stained with ethidium bromide were visualised under a long wave UV source before isolation using either the diethylaminoethyl (DEAE)-cellulose technique as described by Dretzen et al. (1980), or by electroelution as described by Smith (1980).

i. **(DEAE)-cellulose technique:** Strips of DEAE-cellulose paper were prepared by soaking in 2.5M NaCl for 3 hours. They were then washed with 6 volumes of SDW and stored in 1mM EDTA pH 8 at 4°C. Before use, a strip was soaked in 1X electrophoresis buffer for 30min. It was then placed in a slit cut in the gel just in front of the desired band and the band electrophoresed in to the DEAE cellulose paper. After the DNA had adsorbed onto the paper, as judged by examination under long wave UV, the paper was rinsed in SDW and eluted for 3-4 hours into High Salt Buffer (20mM Tris-HCl pH 7.5, 1mM EDTA, 1.5M NaCl) at 37°C with occasional vortexing. At the end of this period, the paper was examined under long wave UV to check that elution had occurred. The solution containing the eluted DNA was extracted once with buffered phenol:chloroform (1:1), three times with water saturated n-butanol and precipitated with ethanol. It was necessary to wash the resultant pellet twice with 70% ethanol, or to re-precipitate the DNA, in order to remove salt carried over from the elution buffer.

ii. **Electroelution of DNA:** A slice of agarose containing the desired DNA fragment was excised from the electrophoresis gel and placed in a dialysis bag with 0.5X TBE. The dialysis bag was sealed without trapping air bubbles and immersed in a shallow layer of 0.5% TBE in an electrophoresis tank. The DNA was then eluted out of the gel by passing a current of 100V through the bag for 1-2 hours, (the migration of the DNA was confirmed by analysis of the dialysis bag containing the gel slice under long wave UV). The polarity of the current was reversed for 1min to release the DNA from the wall of the dialysis bag. The buffer was then removed from the dialysis bag and the DNA purified by three

phenol-chloroform extractions followed by three n-butanol and four ether extractions. The DNA was then collected by ethanol precipitation.

(C4) "Filling-in 5' ends. The DNA fragment (0.5-1 ug) was redissolved in 40ul SDW and 5ul of 10X DNA polymerase buffer and 1ul each of 2mM dATP, dCTP, dGTP and dTTP were added. After addition of 1 unit of E.coli polymerase I (Klenow), the solution was incubated at 37°C for 30min. The DNA was extracted with phenol/chloroform and precipitated with ethanol before use in ligation reactions.

(C5) Phosphatase treatment. Restricted vector DNA fragments were treated with calf intestinal alkaline phosphatase (CIP), essentially as described by Maniatis et al., (1982). Restriction enzyme digests (0.5-1 ug DNA) were extracted with phenol/chloroform, ethanol precipitated and redissolved in 1X CIP buffer. CIP (1 unit) was added and the reactions incubated at 37°C for 1 hour. DNA was extracted five times with phenol/chloroform to inactivate the CIP, washed five times with water-saturated diethylether, ethanol precipitated and then used in ligation reactions.

(C6) Ligation of restriction fragments. Intermolecular ligations typically contained 50ng of vector DNA, 200ng of insert DNA in 1X ligation buffer in a 20ul volume. Reactions were incubated with one unit of T4 DNA ligase at 14°C for 12-16 hours. Intramolecular ligations were performed similarly but in a larger reaction volume (50ul).

(C7) E.coli hosts, preparation and transformation of competent cells. The following E.coli K-12 strains were used depending on which vector DNAs were being employed: Strain JM101 for M13 mp18, M13 mp19 (Norrander et al., 1983) and bluescript (Stratagene) vectors. Strain CJ236 (Kunkel et al. 1987) with M13 mp18 and M13 mp19 when it was necessary to obtain uracil-containing single-stranded DNA for site-directed mutagenesis. Strain JM83 was used with the pPM1

based vectors (Ahlquist and Janda, 1984.) A dam$^-$ E.coli strain, GM 242, was used when dam methylation-free DNA was required.

i. **Preparation of competent cells:** A 5ml culture of JM101, CJ236, JM83 or GM2159 in L-broth was grown overnight at 37^{o}C, (for CJ236, chloramphenicol was added to a concentration of 30ug/ml since plasmid pCJ105[Cm^r] carries the information for pili construction). The next day, 400ul of culture was used to inoculate 40ml of L-broth. The culture was incubated at 37^{o}C in an orbital shaker until exponential phase was reached (usually 2.25 hours). The culture was then chilled on ice, centrifuged (3,400 rpm in a Sorvall RT6000 centrifuge for 5min at 4^{o}C) and the pellet gently resuspended in 20mls of ice-cold 50mM $CaCl_2$. After incubation for 30min in ice, the cells were re-centrifuged (as above) and suspended in 2ml of ice-cold 50mM $CaCl_2$. Competent cells were then used for transformation 2-48 hours later.

ii. **Plasmid transformations:** 200ul of competent cells were gently mixed with 10ul of ligated DNA and left on ice for 30-60 min before application of a heat shock (42^{o}C for 2.5 min). 500ul of L-broth was added and the cells incubated at 37^{o}C in an orbital shaker for 45-60 min before plating out on L-agar plates containing 125ug/ml carbenicillin. For bluescript plasmid transformations, L-agar plates contained 50ug/ml X-GAL (5-bromo-4-chloro-3-indolyl-ß-galactoside) in addition to carbenicillin (125ug/ml). Plates were incubated overnight at 37^{o}C and the colonies were screened by "minipreps" of plasmid DNA (section 2.2 C8). After transformation with bluescript plasmid, the colonies were initially screened by colour selection before "miniprep" analysis.

ii. **Transfection with M13 replicative form DNA:** Either JM101 or CJ236 competent cells were mixed with ligation mixes containing the replicative form DNA on ice for 30min prior to a 42^{o}C heat shock for 2.5min. The transformed cells were left at room temperature for 15min. An overnight JM101 culture was

taken and 100ul added to 3ml of melted soft agar containing 200ug [w/v] isopropyl-ß-D-thio-galactopyranoside and 800ug [w/v] X-GAL, maintained at 45°C in a heated metal block. The transformation mix was added and the mixture overlayed on to plates containing hard agar and allowed to set for 30min before overnight incubation at 37°C. White plaques were screened by "mini rf prep" analysis of the replicative form DNA (section 2.2 C8).

(C8) Rapid small-scale preparation of DNA. A modified version of the method described by Ish-Horowitz and Burke (1981) was used to obtain small amounts of plasmid DNA or M13 replicative form DNA for the purpose of screening recombinant clones. Part of a colony, whose plasmid DNA was to be "minipreped", was used to inoculate 1.5ml of L-broth (plus any antibiotics as appropriate) and incubated overnight at 37°C with shaking. The overnight culture was transferred to a 1.5ml microfuge tube and the cells harvested by centrifugation (10,000X g for 1min at room temperature). For M13 "mini rf preps" 2ml aliquots of a 1/20 dilution an exponential JM101 culture were inoculated with single M13 plaques by means of a sterile toothpick. The cultures were shaken at 37°C for 5 hours and the cells harvested as above.

The cells were resusupended in 100ul of solution I (25mM Tris-HCl pH 8, 10mM EDTA, 50mM glucose). 200ul of freshly made solution II (0.2M NaOH, 0.1% SDS) was added and the mixture incubated for 5min on ice. The solution was neutralised by the addition of 150ul precooled solution III (3M potassium acetate, 11.5% glacial acetic acid) and the mixture incubated for a further 5min on ice. The precipitated protein and chromosomal DNA were removed by centrifugation (10,000xg for 5min). The supernatant was taken and the DNA precipitated with an equal volume of isopropanol. The final pellet was washed with 70% ethanol, dried under vacuum and redissolved in 50ul of SDW of which 5ul was sufficient for analysis with restriction enzymes.

(C9) Large scale preparation of plasmid DNA. Large scale preparations of plasmid DNA were made using a modification of the alkali-lysis method described by Birnboim and Doly (1979). The bacterial clone containing the plasmid to be prepared was grown overnight in 5ml of L-broth. 1ml of the overnight culture was used to inoculate 250ml of L-broth which was incubated for 24 hours in an orbital shaker at 37°C. Cells were harvested by centrifugation at 5,000rpm in a Sorvall GSA rotor. The cells were resusupended in 5mls of solution I (section 2.2 C8) plus lysozyme (1mg/ml), transferred to 50ml polyallomer tubes (Beckman SW27) and incubated for 5min at room temperature. 10mls of solution II (section 2.2, C8) was added and the mixture incubated for 10min on ice, then 7.5mls solution III (section 2.2, C8) added and the mixture incubated for a further 10min on ice. The tubes were then subjected to a clearing spin of 20,000rpm in a Sorvall SS34 rotor at 4°C for 30min. The supernatant was precipitated with 0.7 volumes of isopropanol and the pellet washed with 70% ethanol, dried and resuspended in 5.5ml of 1X TE. CsCl (5.9g) was dissolved in each tube before the addition of 200ul 10mg/ml ethidium bromide. The plasmid DNA was centrifuged to equilibrium at 55,000rpm in a Sorvall TFT65.13 rotor for 16 hours at 15°C followed by 5 hours at 35,000rpm. Plasmid DNA (lower band) was visualised under UV light and removed using a hypodermic syringe. The DNA was washed four times with water-saturated butanol to remove ethidium bromide and dialysed agaisnt 1X SSC overnight at 4°C to remove the CsCl. The DNA was precipitated by the addition of ethanol to 70% and resuspended in TE. The concentration of plasmid DNA was measured spectrophotometrically using $E_{260}^{0.1\%}=20$, and stored at 1mg/ml at -20°C.

(C10) Site-directed mutagenesis.

i. **Production of uracil-containing template DNA:** Oligonucleotide directed mutagenesis was performed by the method of Kunkel (1987). A DNA fragment containing the sequences to be mutagenised was subcloned into the replicative

form of M13mp19 or M13mp18. Recombinant phage were then used to infect E.coli CJ236. 50ul of an over-night culture of CJ236 was used to inoculate 100ml L-broth plus chloramphenicol (30ug/ml). After incubation at 37°C for six hours with shaking, 1-3 X10^6 phage were added and incubation allowed to proceed overnight. The E.coli cells were removed by centrifugation at 10,000rpm for 10min and the 'phage purified from the supernatant by precipitation with 1/4 volume of 3.5M ammonium acetate, 20% PEG 6000. It was confirmed that the purified 'phage had uracil-containing viral DNA by observing a 10^4-fold lower titration of the 'phage on the E.coli strain JM101 compared to CJ236. The template DNA was then purified from the 'phage by phenol extraction, (2.2 C11).

ii. **Synthesis of mutagenic complementary DNA strand:** The oligonucleotide containing the sequence of the desired mutation(s) was phosphorylated by the procedure of Zoller and Smith (1983). 200pmol of oligonucleotide was phosphorylated with 4.5units of T4 polynucleotide kinase in P buffer (100mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 5mM DTT and 430uM ATP), for 45min at 37°C. The reaction was stopped by heating at 65°C for 10min. Phosphorylated oligonucleotide (3 pmole) was then annealed to the uracil-containing template DNA (0.2 pmole) in annealing buffer (20mM Tris-HCl pH 7.4, 2mM $MgCl_2$, 50mM NaCl) at a total reaction volume of 10ul. The annealing was performed by boiling the mixture for 1min, transferring to a waterbath at 70°C and allowing the temperature to fall to 30°C at 1°C/min. The reactions were then placed on ice. The annealed mutagenic primer was then used to prime second-strand synthesis. To the annealed reaction on ice was added 1ul of each of 10X synthesis buffer (5mM dATP, 5mM dCTP, 5mM dGTP, 5mM dTTP, 10mM ATP, 100mM Tris-HCl pH 7.4, 50mM $MgCl_2$, 20mM DTT), T4 DNA ligase (3units/ul) and T4 DNA polymerase (1unit/1ul). The second strand synthesis reaction was then incubated at 0°C for 5min, 25°C for 5min and 37°C for 90min. To stop the reaction 40ul TE was added. To monitor DNA

synthesis a 10ul aliquot was analysed on a 0.8% agarose gel containing TBE buffer. If second strand synthesis and ligation had occured giving double-stranded covalently-closed forms, 10ul of the second strand synthesis mix was used to transfect competent JM101, (2.2 C7ii).

iii. Screening of clones: A number of white plaques was selected for further analysis. Since mutagenic oligonucleotides were designed so that mutagenesis created a novel restriction site, M13 clones carrying the desired mutation were identified by restriction digest mapping of their replicative form DNA. The presence of the mutation was confirmed by dideoxy sequencing of the mutagenised region (see 2.2 C12).

(C11) Preparation of single-stranded DNA. This was carried out essentially as described by Messing (1983). 1.5ml aliquots of an E.coli JM101 overnight culture diluted 1:100 into L broth were inoculated with single colourless M13 plaques by means of sterile toothpicks. The cultures were shaken at 37^{o}C for 4.5-5 hours and then transferred to 1.5ml microfuge tubes and centrifuged at 10 000 xg for 10min. Single stranded template was obtained by precipitation of 800ul of the supernatant with 200ul of 20% polyethyleneglycol 6000, 2.5M NaCl for 30min at room temperature. The phage were harvested by centrifugation for 15min and as much of the supernatant as possible removed. The pellet was re-dissolved in 100ul TE and DNA extracted by vortexing with 50ul of buffered phenol. The mixture was left standing a room temperature for 10min before vortexing once more. The phases were separated by centrifugation at 10 000 xg for 5min. The nucleic acids were precipitated from the aqueous phase by the addition of ethanol and sodium acetate to 70% and 0.4M respectively. The DNA pellets were washed with 100% ethanol, dried and resusupended in 30ul of TE.

Template preparations from Bluescript recombinant clones were perfomed as described above except that one colony was used to inoculate 3mls of L broth and

this was grown for 2.5-3 hours at 37°C before addition of 100ul of R408 helper phage (Stratagene). The culture was then incubated over night, the single stranded DNA extracted as above and finally redissolved in 30ul TE.

(C12) DNA sequencing. The chain termination method of Sanger et al. (1977) was used to determine the sequence of recombinant clones. All reactions were carried out essentially as described in the Amersham sequencing manual using 10uCi of [^{35}S]dATP per clone.

2.2(D) Purification of E.coli RNA polymerase and in vitro transcription.

(D1) Polymerase purification. E.coli RNA polymerase was purified essentially as described by Burgess and Jendrisak (1975). E.coli K12 strain JM83 was grown in 1 litre batches in L-broth and harvested at 3/4 log phase (A_{550} 0.8-0.9). Approximately 1-1.5g (wet weight) of cells were obtained from each litre of exponential culture. The cells (30-40g wet weight) were resuspended in grinding buffer (200ml; 50mM Tris-HCl, pH 7.9, 5%[v/v] glycerol, 2mM Na_2EDTA, 0.1mM DTT, 1mM 2-mercaptoethanol, 0.233M NaCl, 23ug/ml PMSF) containing lysozyme (0.5mg/ml) and incubated on ice for 20mins. The lysed culture was then sonicated using a 10mm probe (100W; Rapidis Model 180) until the viscosity of the lysate was greatly reduced. The temperature of the culture was kept below 10°C at all times. The lysate made up to 475ml total volume with TGED (10mM Tris-HCl, pH 7.9, 5%[v/v] glycerol, 0.01mM Na_2EDTA, 0.1mM DTT) containing 0.2M NaCl and 23ug/ml PMSF. Cellular debris was removed by centrifugation (16 000 xg for 20min at 5°C, Sorvall RC-5) and a 10%[v/v] solution of polyethylimine (pH 7.9) was added to the supernatant to give a final concentration of 0.35%. The polyethylimine precipitate was collected by centrifugation (4 000 xg for 1min at 5°C, Sorvall RC-5) and extracted with 400ml TGED containing 0.5M NaCl and 23ug/ml PMSF. The pellet was collected by centrifugation (as above) and

re-extracted with 300ml of TGED containing 1M NaCl, re-centrifuged (as above) and the supernatant brought to 50% saturation with ammonium sulphate. The precipitate was collected by centrifugation (34 850 xg for 30min at 5°C, Sorvall RC-5) and dissolved in TGED to give a conductivity equal to TGED containing 0.15M NaCl (approximately 60ml).

The sample was loaded onto a 35ml double-stranded DNA cellulose column at 5°C. The column had been prepared according to Alberts and Hendrick (1971) with the modifications of Burgess and Jendrisak (1975). After thorough washing with TGED containing 0.15M NaCl, the bound protein was eluted with a 0.15-1.0M linear NaCl gradient at a rate of 25ml/hour.

Active fractions (as determined be enzyme assay) were pooled and precipitated with ammonium sulphate as before and further purified on a Superose 6 FPLC gel filtration column as described by Buttner et al. (1988). The ammonium sulphate precipitate was dissolved in 50mM NaH_2PO_4 (pH 7.0), 0.1mM Na_2EDTA, 0.1mM DTT, 150mM NaCl, 5%(v/v) glycerol to a final protein concentration of 20-25mg/ml. Particulate matter was removed by centrifugation (10,000xg for 10min at 5°C). Aliquots (200ul) of the protein were applied to a Superose 6 FPLC gel filtration colume (10 x 300mm, Pharmacia plc) equilibriated in 50mM NaH_2PO_4 (pH 7.9), 0.1mM Na_2EDTA, 0.1mM DTT, 500mM NaCl, 5%(v/v) glycerol. The protein was eluted in the same buffer at a flow rate of 0.18ml/min and was collected in 0.5ml fractions. Active fractions were dialysed for 24hours against storage buffer (10mM Tris HCl pH 7.9, glycerol 50% [v/v], Na_2EDTA 0.1mM, NaCl 0.1mM and DTT 0.1mM).

Purification was monitored by enzyme assay (section 2.2 D2) and by SDS-polyacrylamide gel electrophoresis (section 2.2 D4).

(D2) RNA polymerase assay. RNA polymerase was assayed according to Burgess (1969) except that [5,6-^{3}H]uridine 5'-triphosphate (500Ci/mol) was used in place

of [^{14}C]adenosine 5'-triphosphate, and the assay volume was reduced to 100ul. In addition, assays were incubated at 30^{o}C and precipitated counts were filtered and dried on Whatman GF/C filters and counted by liquid scintillation counting.

One activity unit of enzyme encorporates 1nmole of UMP in 10min of incubation under the conditions described.

(D3) Protein assays. Protein assays were carried out according to Bradford (1976) using bovine serum albumen as reference.

(D4) Protein analysis by gel electrophoresis. Proteins were fractionated on SDS-polyacrylamide (10%)/bisacrylamide (0.025%) slab gels with a 3% acrylamide, 0.08% bisacrylamide stacking gel using the buffer system of Laemmli (1970). A small scale gel system was used, the resolving gel poured from a 9.75ml acrylamide mix and the stacking gel from a 6ml acrylamide mix. Protein samples to be analysed were mixed with 1/3 volume of 4X protein sample loading buffer, denatured by boiling for 3mins and then loaded on the gel. Electrophoresis was carried out at 16mA until the tracking dye reached the end of the gel. Polypeptides were detected by staining with Coomassie blue R250.

(D5) In vitro transcription reactions and analysis of products. Transcription reactions were carried out essentially as described by Ahlquist and Janda (1984). Transcription reactions contained 0.1ug of EcoRI-cut plasmid DNA, 0.02-0.03 units of E.coli RNA polymerase, 0.25 units of RNAsin per ul of reaction mix, 200uM of each ribonucleoside triphosphate, 25mM Tris-HCl pH 8.0, 5mM $MgCl_2$ and 150mM NaCl. Reactions were incubated for a total of 2hours at 37^{o}C. After incubation for 1 hour a further 0.01-0.015 units of E.coli RNA polymerase per ul of mixture were added. Transcription reactions were stopped by phenol/chloroform extraction. The products were collected by ethanol precipitation, washed with 70% ethanol, dried under vacuum and resuspended in SDW at a concentration of 1ug linear DNA template per ul. When necessary, the linear DNA template was removed by LiCl

precipitations as follows: After phenol/chloroform extraction and ethanol precipitation, the products of transcription reactions were resuspended at a concentration of 0.5ug linear DNA template per ul. An equal volume of 4M LiCl was added and the mixture incubated at -20°C overnight. The precipitated single-stranded RNA was then collected by centrifugation (10, 000 xg for 10mins at 5°C), washed with 70% ethanol, vacuum dried and resuspended in SDW.

Samples of the transcription reactions (usually 1ul) and standards of CPMV RNA of known concentration were analysed by electrophoresis on 1% agarose gels containing formaldehyde and the amount of full length transcript quantitated by ethidium bromide fluorescence quantitation (section 2.2 A4).

2.2(E) In vitro translation and analysis of products.

(E1) In vitro translation. Virion and transcript RNAs were translated in vitro in a messenger-dependent rabbit reticulocyte lysate (MDL). Translation mixtures contained 19ul MDL, 1ul of a 1ug/ul solution of RNA, 1ul of a 2M KCl/10mM MgCl mix, 1ul ^{35}S methionine (10uCi), 1ul 0.2M creatine phosphate, 1ul amino acid mix containing 0.5-3mM of each amino acid except methionine. Incubations were for 90min at 30°C and were stopped by the addition of 7ul of a solution of 10% SDS, 1M 2-mercaptoethanol. Samples were then denatured by boiling for 5min or by leaving at room temperature for 60min.

Trichloroacetic acid (TCA)-precipitable radioactivity was measured by adding a 2ul aliquot of the translation reaction to 1ml of bleaching solution (0.5M NaOH, 0.5M H_2O_2) and incubation for 10min at 37°C. Radioactive polypeptides were precipitated on ice for 10min by addition of 0.5ml 25% TCA and collected on Whatman GF/C filters by vacuum filtration. The precipitate was washed twice with 5ml 8% TCA and once with 5ml ethanol. Filters were dried and then counted in 4ml of scintillant (0.5% 2, 5-diphenyloxazole [PPO] in toluene) using a

Beckman LS 7500 liquid scintillation counter.

(E2) Protein gel electrophoresis and fluorography. In vitro translation products were analysed on SDS/Laemmli buffer polyacrylamide gels (see 2.2 D4). Large scale gels were prepared from a 30ml resolving gel mix containing 12.5% acrylamide, 0.1% bisacrylamide; and a 8.7ml stacking gel mix containing 5.7% acrylamide, 0.15% bisacrylamide. Denatured samples were mixed with 4X protien gel loading buffer and loaded directly onto the gel. Electrophoresis was carried out overnight at 50V. When the tracking dye reached the end of the gel electrophoresis was stopped and the gels were fixed for 2-18 hours in 30% ethanol [v/v], 12% acetic acid [v/v]. If gels were not to be fluorograghed, they were washed three times for 30min in dH_20, dried onto Whatman 3MM paper over a boiling water bath under vacuum and exposed to Fuji RX X-ray film at -70°C (Laskey and Mills, 1977). Gel fluorography was carried out by infiltration of the gel with 15% ethanol [v/v], 0.5% PPO [w/v], 55% acetic acid [v/v], 30% xylene [v/v] for 1-2 hours. Gels were then rinsed under a continuous stream of water for 1 hour before drying. Autoradiography was carried out as described above.

2.2(F) Isolation and electroporation of protoplasts.

(F1) Growth of Cowpeas. Cowpea plants for the isolation of protoplasts were grown essentially as described by Hibi et al. (1975). Cowpea seed was germinated on moistened vermiculite in the dark at 26°C for three days. The seed coats were removed from the seedlings and the roots placed in contact with Hoagland's growth media (Hoagland's solution contains per litre: 1.18g $Ca[N0_3].4H_20$, 0.51g $KN0_3$, 0.49g $MgS0_4.7H_20$, 0.016g KH_2P0_4, 30mg FeNa EDTA, 8.6mg $ZnS0_4.7H_20$, 0.25mg $Na_2Mo0_4.2H_20$, 22.3mg $MnS0_4.4H_20$, 0.83mg KI, 6.2mg H_3B0_4, 25ug $CuS0_4.5H_20$ and 25ug $CoCl_2.6H_20$). Plants were propagated in a growth cabinet with 14hours light (18Klx at plant level, supplied by fluorescent tubes and tungsten

bulbs) at 26°C and 10 hours in darkness at 22°C. The primary leaves of 10-11 day old plants were used for protoplast isolation.

(F2) Isolation of protoplasts. Protoplasts were prepared under sterile conditions. The primary leaves were surface sterilized by rinsing in 70% ethanol and immersing in a 5% solution of "Domestos" [v/v] for 8min, followed by three washes in SDW. The leaf mid-ribs were excised and the lower epidermis removed with forceps. Tissue was plasmolysed on 0.6M mannitol for at least 15min and then digested in 2.5% cellulase R-10, 0.25% macerozyme R-10 in 0.6M mannitol at pH 5.5. Digestion proceded for 4-5 hours at 30°C in a shaking water bath at 60 excursions per minute.

Protoplasts were separated from tissue debris by filtration through a 66uM nylon mesh and were washed three times by centrifugation at 100g for 4min and resuspension in 0.6M mannitol. Protoplasts were resuspended in a known volume of 0.6M mannitol and counted using a haemocytometer.

(F3) Electroporation of protoplasts. Freshly isolated protoplasts were resuspended at $1x10^6$/ml and electroporated with virion RNA (1ug unfractionated RNA or 1ug M RNA plus 1ug B RNA /$1x10^6$ protoplasts) or a mixture of in vitro transcripts plus purified B RNA (1-20ug full-length transcripts plus 1ug B RNA /$1x10^6$ protoplasts) or purified B RNA alone (1ug /$1x10^6$ protoplasts); see 2.2(A3i) for details of B RNA purification. Protoplasts were electroporated 1ml at a time, on ice with a single pulse from a 0.05uF capacitor. Protoplasts that had been electroporated with identical RNA solutions were combined, kept on ice for 5min, collected by centrifugation and resuspended in "incubation mix" (1mM KNO_3, 1mM $MgSO_4.7H_2O$, 0.2mM KH_2PO_4, 10mM $CaCl_2$, 1uM KI, 10nM $CuSO_4$, 0.6M mannitol, 25ul/ml gentamycin and 1ug/ml [2,4-D]; Rottier et al. 1979) at $5x10^5$ electroporated protoplasts/ml. Protoplasts were cultured in 2ml aliquots in 10ml Sterilin tubes at 25°C with continuous lighting at 2.5Klx.

2.2(G) Analysis of electroporated protoplasts.

(G1) Determination of protoplast viability. Protoplast viability was assessed by mixing a 50ul aliquot of resuspended protoplasts with an equal volume of phenosafranine prepared at 0.1%[w/v] in 0.6M mannitol (Widholm, 1972). Protoplasts were counted using a haemocytometer. Non-viable protoplasts did not exclude the phenosafranine and appeared red. At least 200 protoplasts were counted in each sample.

(G2) Infectivity assay by fluorescent antibody staining. To assess the percentage of protoplasts that become infected after electroporation, the indirect method of fluorescent antibody staining was used (Maule et al., 1980). A small sample of protoplasts was washed with 0.6M mannitol, spread on a microscope slide and quickly dried with a hair dryer. The protoplasts were fixed in acetone for 30min and either processed immediately or kept dry at $4^{o}C$. Slides were rinsed with water and reacted with antiserum raised in rabbit against CPMV virions. The antiserum was diluted 1:800 in PBS before use. Slides were incubated for 1 hour at room temperature in a high humidity sealed box. After gently washing in PBS for 30min, the slides were reacted with goat anti-rabbit immunoglobulin labelled with fluorescein (Wellcome Reagents Ltd.) at a dilution recommended by the supplier (usually 1:40) for 30min under the same conditions as for the first antibody reaction. After washing in PBS for 30min the slides were mounted in 90% glycerol buffered with 10mM sodium carbonate, pH 8.5, and examined under a Zeiss Universal Microscope equipped with epi-fluorescence. Protoplasts showing a bright yellow-green fluoresence were scored positive whereas non-infected protoplasts showed a dull green fluoresence. At least 200 protoplasts per slide were counted and two slides were prepared for each sample.

(G3) Extraction of nucleic acids from protoplasts. Nucleic acids were extracted from protoplasts as described by de Varennes et al. (1985). Samples of 7.5 $X10^{5}$ to

1 X10^6 protoplasts were collected by centrifugation and the protoplast pellet stored at -20^{0}C. The frozen pellet was resuspended in 400ul of 40mM Tris-HCl pH 8.5, 80mM NaCl, 4mM Na_2EDTA , 5% [w/v] sarkosyl and extracted with 450ul of phenol/chloroform (8:1) equilibrated in the same buffer. The two phases were separated by low speed centrifugation, the aqueous phase collected and the nucleic acids precipitated with 2.5 volumes of ethanol. Nucleic acids were collected by centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in SDW at a concentration of nucleic acids from 1.25 X10^5 protoplasts/4ul.

(G4) Detection of CPMV RNA by Northern blotting. Nucleic acids extracted from protoplasts were analysed on agarose gels containing formaldehyde, see 2.2 (A4). Large scale agarose gels were poured from a 150ml mix. Electrophoresis was at 20mA overnight. The gel was then placed directly on a capillary blotting tank (Southern, 1975) and the nucleic acids transferred to Hybond N (Amersham) using 20X SSC as buffer. Transfer was allowed to proceed overnight. The Hybond N was then wrapped in cling film and placed on a short wave UV transilluminator for 3mins to crosslink the nucleic acids to the Hybond N.

The Hybond N filter was then pre-hybridised, hybridised and washed as described in section 2.2 (B4iii). Two methods were used to produce M RNA-specific probes. The first method was to "oligo-labelling" a HindIII fragment from pPMM2902 (corresponding to nucleotides 482-2211 of the M RNA sequence) with ^{32}P using [α^{32}P]CTP and Klenow as described by Feinberg and Vogelstein (1983). Unincorporated label was separated from the DNA as described in 2.2 (B4ii). The second method was to produce labelled negative sense M RNA molecules. Clone pSPM203 contains a full length cDNA copy of M RNA in the PstI site of the transcription plasmid pSPT18 (Lomonossoff, pers. comm.). After linearisation with SmaI, negative sense transcripts were produced using T7 polymerase and [α-32P]UTP essentially as described by Melton et al. (1984). Unincorporated label

was separated from the RNA by LiCl precipitation (2.2 D5).

(G5) Extraction of proteins from protoplasts. Protoplasts were collected by centrifugation and frozen. Proteins were extracted as described by Wellink et al. (1987a). Frozen pellets corresponding to aliquots of $1x10^6$ protoplasts were resuspended in 100ul HB buffer (50mM Tris-acetate pH 8.2, 10mM potassium acetate, 1mM EDTA, 5mM DTT, 1mM phenylmethylsulphonyl fluoride [PMSF], 10% sucrose) and centrifuged at 4^oC for 30min at 30,000g yielding a supernatant (S30) fraction and a pellet fraction (P30).

(G6) Immunological detection of polypeptides.

i. **Electrophoresis and electroblotting:** The presence of certain proteins in electroporated protoplasts was determined by Western blotting. Proteins from the S30 and P30 fractions were separated on SDS-polyacrylamide gels using a small scale gel system, see 2.2 D(4). Proteins were separated on either 10% or 7.5% polyacrylamide resolving gels containing 0.19% bisacrylamide.

Polypeptides separated by electrophoresis were electroblotted onto nitrocellulose in 25mM Tris-HCl pH 8.3, 192mM glycine, 0.05% SDS 20% methanol at 20V overnight at 4^oC (Towbin et al., 1979).

ii. **Immunological detection:** Immunological detection was essentially as described by Blake et al. (1984). All reactions were carried out on a shaking platform. The nitrocellulose filter (Western blot) was incubated overnight at 5^oC in 1X PBS buffer containing 5% Marvel [Cadburys skimmed milk], 0.1% Tween 20 and antiserum to the appropriate protein. All subsequent incubations were at room temperature. Excess antisera was removed by four washes for 15min in the same buffer solution. The alkaline phosphatase conjugate was diluted into PBS plus 5% Marvel and 0.1% Tween and incubated with the filter for 4 hours. The filter was then washed four times with the same buffer solution. After two further washes for 5min in 0.15M veronal acetate pH 9.6, the proteins were visualised by

incubation in 0.133M veronal acetate pH 9.6, 100ug/ml nitroblue tetrazolium, 50ug/ml 5-bromo-4-chloro-3-indoyl phosphate and 4mM $MgCl_2$.

Two antisera were used, an anti-coat protein antiserum (see 2.2 G2) and an antiserum to the 58K and 48K proteins known as the 58/48K antiserum. The 58/48K antiserum was raised against a synthetic oligopeptide whose sequence corresponds to the C-terminal 14 amino acids of the 48K and 58K proteins of CPMV (see Chapter 1). This antiserum was raised by Drs. N. Huskisson and P. Barker at the Institute of Animal Physiology, Babraham. Prior to use, the 58/48K antiserum was pre-absorbed against protein from healthy cowpea plants to reduce non-specific binding.

iii. **Pre-absorbtion of 58/48K antiserum:** 1ml of the 58/48K antiserum was added to 1ml PBS in a 30ml corex tube on ice. The freeze dried sap from 18g of cowpea "Blackeye" primary leaves was added, 2 large spatula-fulls per hour for five hours. The thick suspension was incubated at 0^oC overnight, centrifuged (16 000 xg for 10min at 5^oC, Sorvall RC-5) and the supernatant collected. The pellet was resuspended in 1ml PBS and incubated at 0^oC overnight. After centrifugation (as previous) the supernatant was collected. The two supernatant fractions, containing the pre-absorbed antibodies, were combined and stored at -20^oC.

iv. **Staining of molecular weight markers:** Polypeptides that were used as molecular weight markers were visualised on Western blots by staining with iodine and starch as described by Kumar et al. (1985). For this, the Western blot was incubated for 2min in a 100ml solution containing 0.1% KI, 0.1M HCl. Chloramine-T (1ml of 5%[w/v]) was added and the blot incubated for a further 2min. The blot was then washed in distilled water, followed by incubation in a 0.1% starch solution until the protein profile had developed fully (usually 2-5 min). The blot was then washed in distilled water and allowed to dry.

Chapter 3: Further Characterisation of 8-14, a temperature sensitive mutant of cowpea mosaic virus.

3.1 Introduction.

The mutant 8-14 was isolated by Evans (1985b) after treatment of CPMV virion RNA with nitrous acid. Initially 8-14 was selected on the basis of maintained aberrant lesion formation when passaged on leaves from Phaseolus vulgaris L. var. "Pinto". Mutant 8-14 induces large, diffuse chlorotic lesions on "Pinto" whereas the wild-type virus produces large necrotic local lesions. Mutant 8-14 is also temperature sensitive (ts) with respect to symptom production on the local lesion host Vigna unguiculata (cowpea) cv. "Early Red" and the systemic host Vigna unguiculata (cowpea) cv. "Blackeye Early Ramshorn". At the permissive temperature (20^{o}C) both wild-type virus and 8-14 induce lesions on inoculated "Early Red" and a chlorosis of inoculated "Blackeye Early Ramshorn". However, at the restrictive temperature (32^{o}C) no symptoms develop after inoculation of mutant 8-14 on either host.

Evans (1985b) carried out a preliminary investigation of 8-14. He demonstrated that mutant 8-14 was completely temperature sensitive (no infectious virus being produced at the restrictive temperature) after inoculation of "Blackeye Early Ramshorn". The genetic lesion was mapped on to the B RNA since the temperature sensitivity was complemented by wild-type B components. The phenotypic lesion in mutant 8-14 appeared to be at the level of virus accumulation rather than virus replication. At the restrictive temperature reduced amounts of RNA were synthesised in 8-14-inoculated "Blackeye Early Ramshorn" leaves but no detectable viral coat proteins or infectious virus produced. This suggested that, although RNA synthesis occurred on a small scale, the RNAs were not encapsidated into infectious virus particles. There are several possible

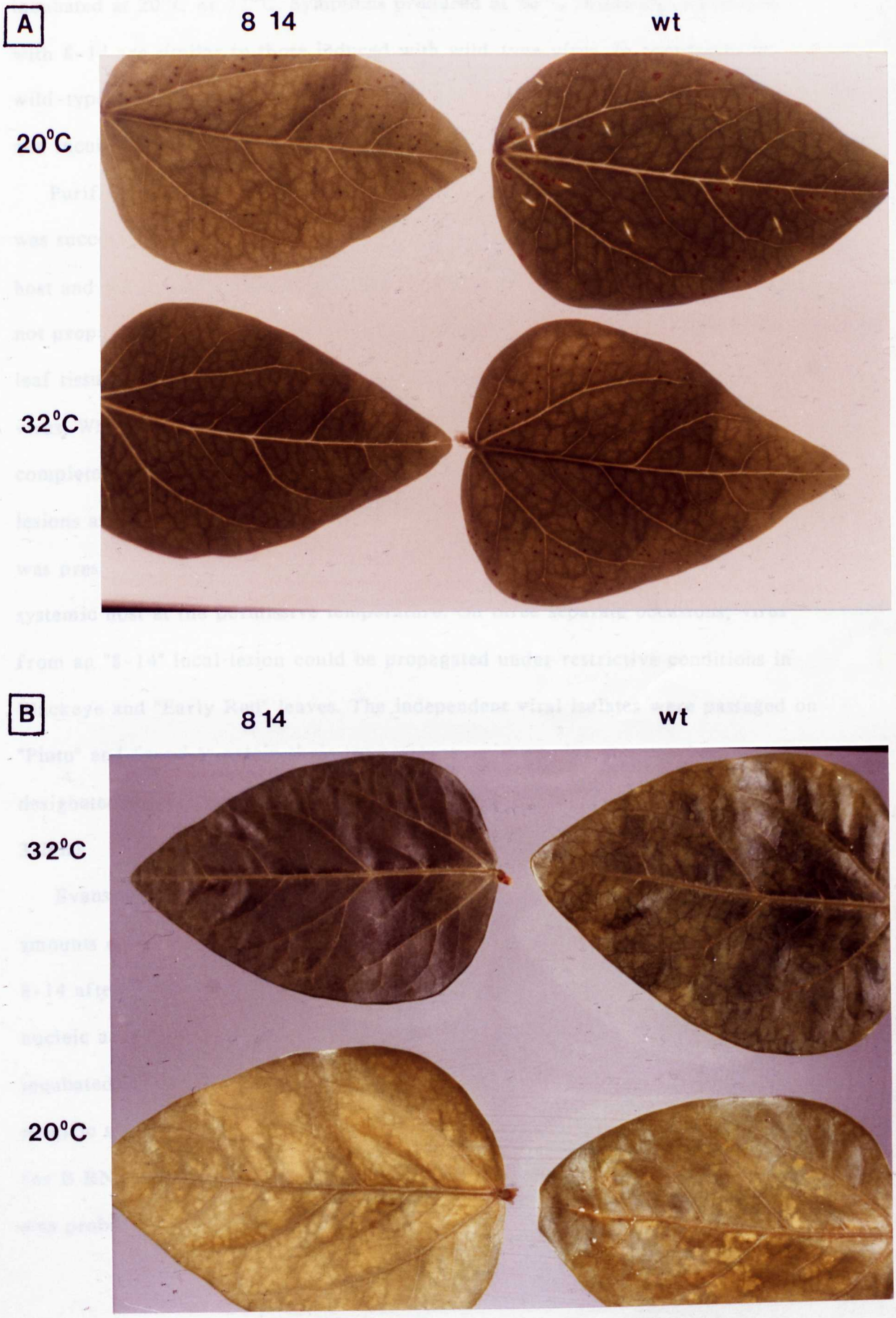
reasons for this. For example, proteins encoded by B RNA are required for proteolytic processing of the M RNA encoded polyproteins resulting in release of the coat proteins. Therefore, a defect in the expression or functioning of the B RNA-encoded 24K protease or 32K putative protease co-factor could be responsible for the observed phenotype.

In this chapter, the biochemical phenotype of mutant 8-14 was further characterised with the aim of locating the temperature sensitive lesion to a particular gene function and correlating this with the change(s) in nucleotide sequence. However, for such an analysis to be successful, it was important that the 8-14 genome contained few nitrous acid induced changes so that location of the genetic lesion responsible for the ts phenotype was feasible. In order to minimise the probability of multiple mutations, the concentration of nitrous acid that was used to induce 8-14 had been carefully selected on the basis of a dose-response curve (Evans, personal communication). However, the extent of the genetic alterations in the genome had not been determined. Therefore, to gain more information about the effect of the mutagen on the genome of 8-14, genomes of mutant, revertant and wild-type virus were compared using the technique of two-dimensional RNA finger-printing.

3.2 Phenotypic characterisation of 8-14.

3.2(A) Symptom production and virus propagation.

As the cowpea cultivar "Blackeye Early Ramshorn" used by Evans (1985b) was not available, the temperature sensitive (ts) phenotype of 8-14 with respect to symptom production on systemic hosts was assayed on cowpea "California Blackeye" and shown to be similar to that reported. The ts phenotype of 8-14 on "Early Red" was also confirmed. Figure 3.1 shows primary leaves detached from Blackeye or "Early Red" plants inoculated with 8-14 or wild-type virus and


A
8 14
wt
20°C
32°C
B
8 14
wt
32°C
20°C

incubated at 20°C or 32°C. Symptoms produced at 20°C following inoculation with 8-14 are similar to those induced with wild-type virus. In contrast to the wild-type situation, no symptoms developed on the leaves inoculated with 8-14 and incubated at the restrictive temperature.

Purification of significant quantities of 8-14 from systemically infected leaves was successful only if single local lesions were used to inoculate the systemic host and the ts nature of the inoculum confirmed (see 2.2 B2). Mutant 8-14 did not propagate as efficiently as wild-type CPMV, a gram of systemically infected leaf tissue yielding 50-250ug of 8-14 virus compared with 1mg for wild-type virus. When assayed on "Early Red", preparations of purified 8-14 were not completely ts, incubation at 32°C resulting in the formation of about 5% as many lesions as were produced at 20°C. Virus infectious under restrictive conditions was presumed to arise by reversion during propagation of the mutant in the systemic host at the permissive temperature. On three separate occasions, virus from an "8-14" local lesion could be propagated under restrictive conditions in Blackeye and "Early Red" leaves. The independent viral isolates were passaged on "Pinto" and found to retain their temperature resistant phenotype and so were designated revertants of 8-14.

3.2(B) Genome replication.

Evans (1985b) determined that, in comparison with the wild-type, reduced amounts of RNA are produced in "Blackeye Early Ramshorn" leaves infected with 8-14 after incubation at the restrictive temperature. To clarify this observation, nucleic acids were phenol extracted from equal fresh weights of Blackeye leaves incubated at 20°C and 32°C and were spot hybridised and probed for CPMV-specific sequences (Figure 3.2). The dot blot presented in Figure 3.2 was probed for B RNA, essentially identical results were obtained when a duplicate dot blot was probed for M RNA.

FIGURE 3.2.

Dot blot hybridisation analysis of RNA from phenol extracts of "California Blackeye" leaves.

Each pair of leaves was inoculated with either:-

A. wild-type CPMV.

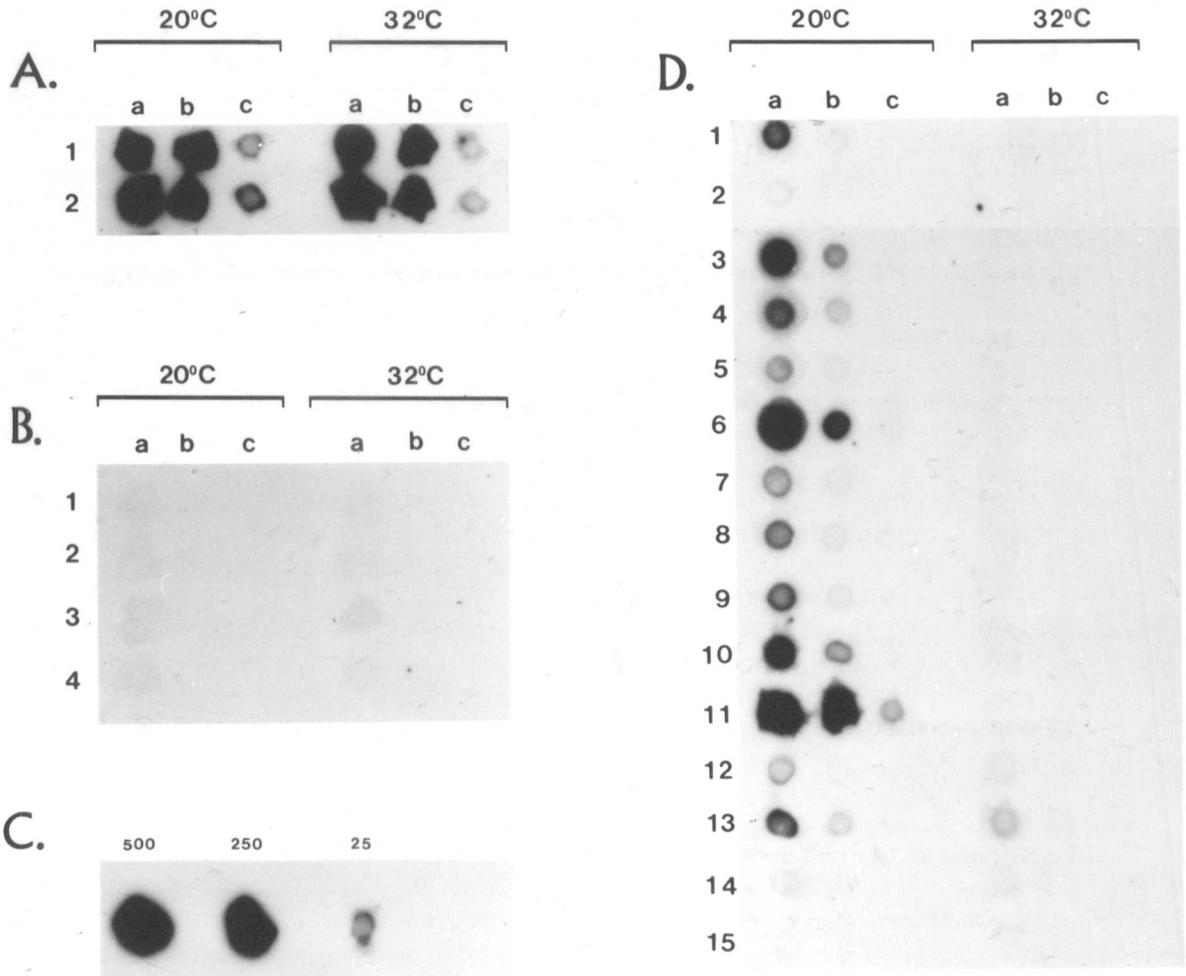
B. mock inoculated.

D. mutant 8-14.

and one member of each pair incubated at 20°C and the other at 32°C.

The nucleic acids in leaves that had been incubated at 20°C or 32°C (as indicated above the panels) were probed for the presence of CPMV B RNA. The letters above the panels indicate the dilution of the nucleic acids applied to each dot, **a**, 1/1 (corresponding to nucleic acids from 150mg fresh weight leaf); **b**, 1/2.5; **c**, 1/40. The numbers to the left of the panels indicate individual leaf pairs.

In panel C, purified virion B RNA (ng, corresponding to the numbers above the dots) has been applied to each dot.


A.
20°C
32°C
a b c a b c
1
2
B.
20°C
32°C
a b c a b c
1
2
3
4
C.
500
250
25
D.
20°C
32°C
a b c a b c
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

The accumulation of viral RNA in Blackeye leaves infected with wild-type virus is independent of, and not significantly affected by, temperature of incubation (Figure 3.2 A). This contrasts with the RNA accumulation in the fifteen Blackeye leaf pairs inoculated with 8-14 (Figure 3.2 D). In twelve of the leaves incubated at 20°C viral RNA is detected (Figure 3.2 D; 1 and 3 to 13), the degree of RNA accumulation varies but is generally less than wild-type. In the remaining three Blackeye leaves (Figure 3.2; 2,14 and 15) no significant accumulation of RNA could be detected. Since each pair of Blackeye leaves was inoculated with virus from a single "Pinto" local lesion, variation in the level of viral RNA accumulation may reflect differing amounts of virus in isolated local lesions. The reduction in RNA accumulation in 8-14 infected leaves under permissive conditions, when compared to wild-type, implies that the multiplication cycle is affected at the permissive as well as the restrictive conditions as a result of the induced mutation(s).

There are low levels of hybridisation corresponding to nucleic acid samples from leaves inoculated with 8-14 and maintained under restrictive conditions. However, in all but one case (Figure 3.2 D; 13), a similar level of hybridisation is associated with the nucleic acid samples from mock inoculated leaves (Figure 3.2; B). This hybridisation is due to non-specific binding of the radioactive probe.

This data indicates that 8-14 RNA does not accumulate significantly above background levels in "Blackeye Early Ramshorn" leaves under restrictive conditions.

3.2(C) Genome expression.

A preliminary investigation into genome expression was carried out by translating wild-type and mutant RNAs <u>in vitro</u> at 32°C, (Figure 3.3). The variation in intensity is a reflection of unequal loading and is not due to differences in efficiency of translation.

FIGURE 3.3.

<u>In vitro</u> translation products of wild-type and 8-14 RNAs at the restrictive temperature.

Products from the translation of:-

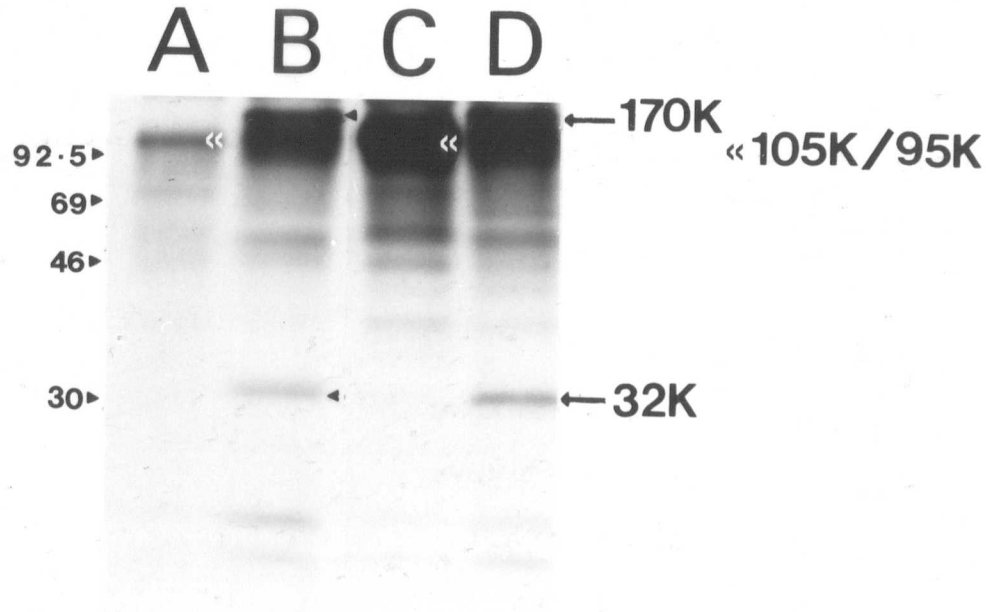
A. Wild-type M RNA.

B. Wild-type B RNA.

C. 8-14 M RNA.

D. 8-14 B RNA.

Products were analysed on a 12.5% polyacrylamide/SDS gel. The size of the protein markers used (Mr X 10^{-3}) is indicated on the left hand side. The positions of the 170K and 32K proteins are indicated with black arrows. The position of the co-migrating 105K and 95K proteins is indicated with white arrows.


A
B
C
D
92·5
69
46
30
170K
105K/95K
32K

Wild-type M RNA is translated to give the 105K and 95K proteins which have not been resolved in the gel system used. The M RNA purified from 8-14 is translated to give similar products. Under the conditions of the M RNA in vitro translations, ie in the absence of B RNA encoded proteins, these primary translation products undergo no further cleavages.

Upon in vitro translation of wild-type B RNA, the primary cleavage products of 170K and 32K can be seen. Proteins of equivalent size are also directed by B RNA from 8-14. This result shows that the translation of 8-14 B RNA and the first proteolytic cleavage occurs in vitro at the restrictive temperature.

3.3 Genetic Characterisation.

In order to determine the level of genetic alteration induced by the nitrous acid process used to produce 8-14, fingerprints of wild-type and mutant genomes were produced. Since the ts lesion in 8-14 had previously been mapped to the B-RNA (Evans, 1985b), fingerprinting was initially limited to the RNA from the bottom components of 8-14 and the parental wild-type CPMV.S2. The results are presented in Figure 3.4.

A typical fingerprint resolved about thirty slower-migrating "diagnostic" oligonucleotides in the size range of 10 to 25 bases, representing about 8% of the total B RNA sequence. Problems were encountered consistently resolving the diagnostic oligonucleotides, reflecting the inefficient kinase labelling of these larger oligonucleotides. Only those diagnostic oligonucleotides that were consistently resolved are discussed. Fingerprints of 8-14 B RNA and CPMV.S2 B RNA are similar, the oligonucleotides X1-X5 being labelled to allow orientation (Figure 3.4, A and B). Only four changes (the additional spots Y1, Y2, Y3 and Y4) were consistently resolved in the diagnostic oligonucleotides. A further four oligonucleotides (labelled in the box Z) are unique to the 8-14 RNA fingerprint

FIGURE 3.4.

Two dimensional RNA fingerprints of B RNAs from CPMV and 8-14.

Fingerprints of:-

A. 8-14 B RNA.

B. Wild-type CPMV.S2 B RNA.

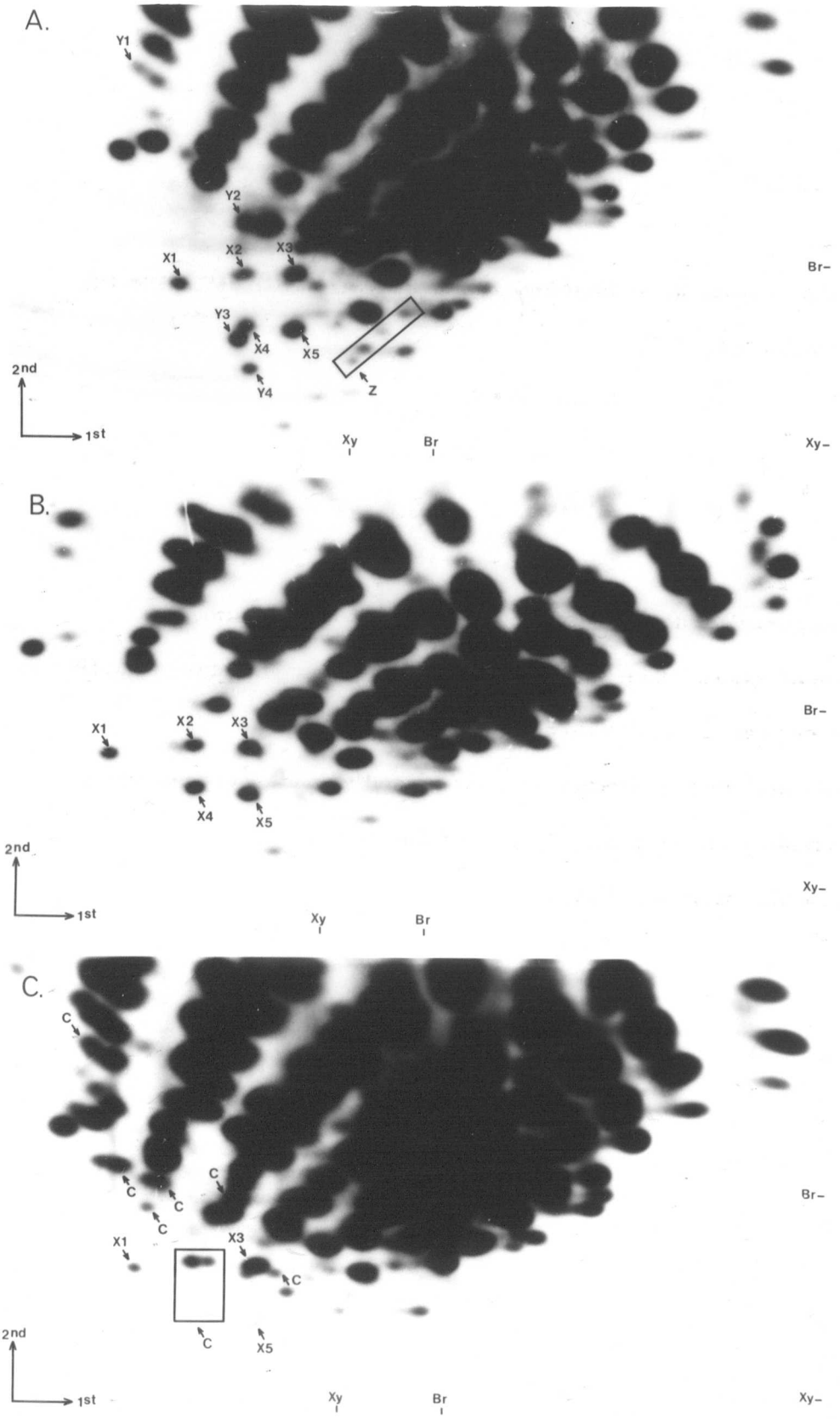
C. CPMV.S1 B RNA.

The oligonucleotides X1-X5 are labeled to allow orientation.

Oligonucleotides labeled Y, Z and C are not conserved between the fingerprints being compared (for details see text).

Arrows indicate the direction of the first and second electrophoresis.

The mobility of the marker dyes bromophenol blue (Br) and xylene cyanol (Xy) in each dimension is indicated.


A.
Y1
Y2
X1
X2
X3
Br-
Y3
X4
X5
Z
Y4
2nd
1st
Xy
Br
Xy-
B.
Br-
X1
X2
X3
X4
X5
2nd
1st
Xy
Br
Xy-
C.
C
C
C
C
C
C
Br-
X1
X3
C
C
X5
2nd
1st
Xy
Br
Xy-

presented in Figure 3.4 but since corresponding spots were not detected on other mutant fingerprints their relevance remains unknown. They may possibly be partial products of the T1 digestion.

The changes resolved between wild-type and mutant fingerprints are either nitrous acid induced or evolutionary. The possibility of alterations due to evolution can not be ignored since RNA populations are heterogeneous and known to evolve rapidly (Holland et al,, 1982). To gain more information on the level of genetic alteration resolved by the RNA fingerprinting technique, a fingerprint of a second isolate of the virus (CPMV.S1) was prepared and compared with that of CPMV.S2, Figure 3.5, B and C). (For the origin of the two isolates, see section 2.2 A1.).

When compared with B-RNA from CPMV.S2, the fingerprint of CPMV.S1 reveals at least eight changes. The oligonucleotides X1, X3 and X5 have been labeled on the fingerprint of CPMV.S1 to allow orientation. It is not however possible to definitely label spots corresponding to X2 and X4, X4 being absent and two oligonucleotides migrating to the position associated with X2 (see Figure 3.5, C; boxed area). In addition to the changes within the boxed area, six additional oligonucleotides are indicated by the letter "C" on the fingerprint of CPMV.S1.

Using the RNA fingerprinting technique, the B RNA from CPMV.S2 is more closely related to a nitrous acid induced mutant derived from it than to a separate isolate of the virus. This shows that, compared with evolutionary variability, the mutagenic treatment has caused relatively few genetic alterations.

When producing mutants by random mutagenesis it is desirable to keep silent base changes to a minimum. The striking similarities maintained between wild-type and mutant B RNAs implies that the 8-14 genome contains relatively few mutations. In order to investigate this further, fingerprints of wild-type and

FIGURE 3.5.

Two dimensional RNA fingerprinting of M RNAs from CPMV.S2 and 8-14.

Fingerprints of:-

A. Wild-type CPMV.S2 M RNA.

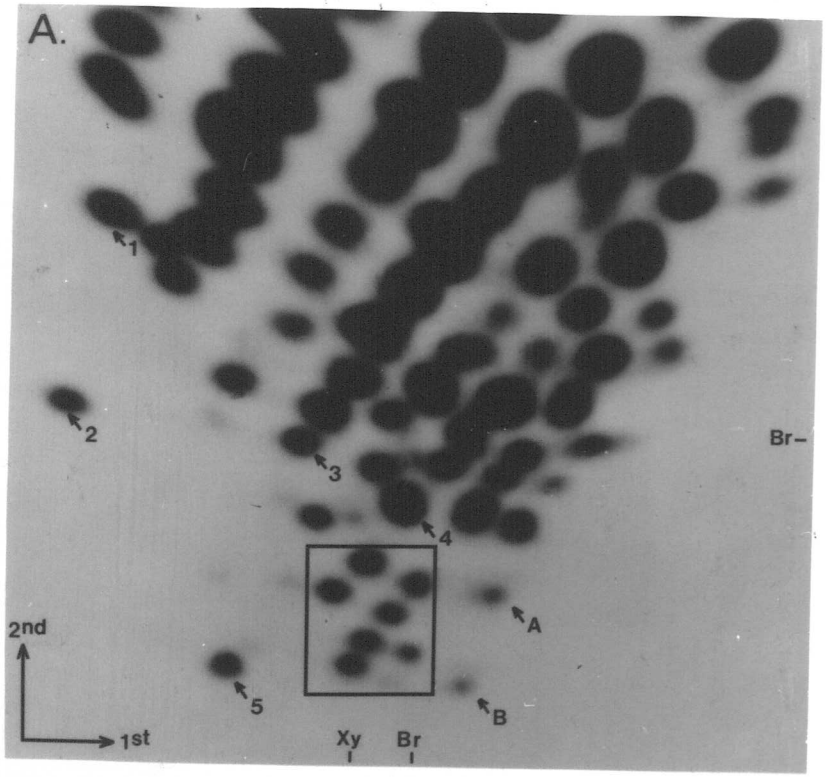
B. 8-14 M RNA.

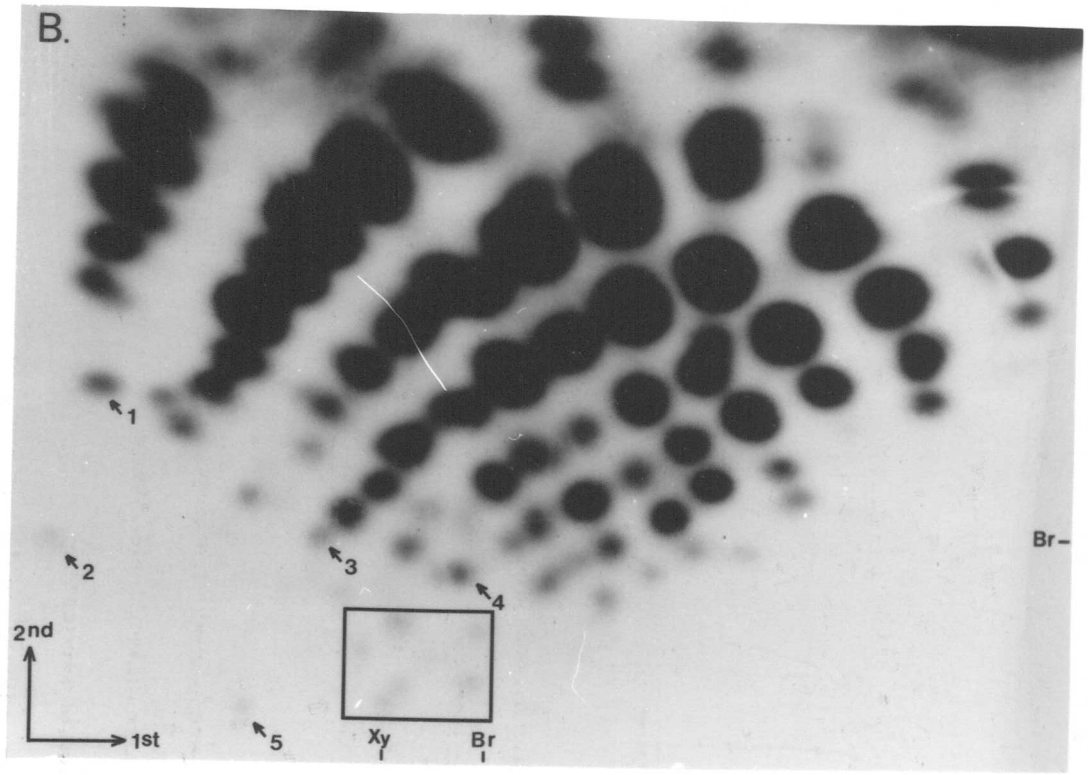
The boxed oligonucleotides and those labeled 1-5 allow orientation.

Oligonucleotides labeled A and B are not conserved between the fingerprints (for details see text).

Arrows indicate the direction of the first and second electrophoresis.

The mobility of the marker dyes bromophenol blue (Br) and xylene cyanol (Xy) in each dimension is indicated.


A.
1
2
3
4
5
A
B
Br–
2nd
1st
Xy
Br


B.
1
2
3
4
5
Br–
2nd
1st
Xy
Br

mutant M RNAs were prepared; since the mutant 8-14 was isolated after mutagenesis of virion RNA and yet the ts lesion maps only on to the B RNA, the level of variation between the M RNAs will illustrate the level of silent mutation. See Figure 3.5.

The fingerprints of mutant and wild-type M RNAs were not produced under identical electrophoretic conditions and so corresponding oligonucleotides have a slightly altered migration profile. However a careful comparison reveals that all oligonucleotides resolved on the mutant M RNA fingerprint are present on the wild-type M RNA fingerprint. To allow orientation, five oligonucleotides have been labeled and a boxed region included. The two oligonucleotides (A and B) resolved only on the wild-type fingerprint are thought not to be detected on the mutant profile due to inefficient kinase labelling of the larger oligonucleotides. Fingerprinting of mutant and wild-type M RNAs therefore reveals no changes indicating that the level of silent mutation in the 8-14 genome is low.

Having established that the fingerprinting technique resolved changes between wild-type and mutant B RNAs and that the level of silent mutation in the 8-14 genome was low, I endeavoured to see if it would be possible to locate the genetic alteration(s) connected with the ts phenotype. To achieve this, three independent revertants of 8-14 were isolated and their B RNAs fingerprinted. By comparing mutant and revertant fingerprints, it was hoped to reveal an alteration in the genetic profile associated with the ts^+ to ts^- reversion. This information might also allow the ts lesion to be mapped on to the B RNA. In Figure 3.6 the fingerprint of revertant R2 is presented and a comparison with the fingerprint of 8-14 reveals that the four changes (Y1-Y4) specific to the mutant profile (when compared to the wild-type) are also resolved in the revertant fingerprint. The fingerprints of the three independent revertants were identical and no additional changes could be resolved between revertant and mutant fingerprints. This result

FIGURE 3.6.

Two dimensional RNA fingerprints of B RNAs from 8-14 and revertant R2.

Fingerprinting of:-

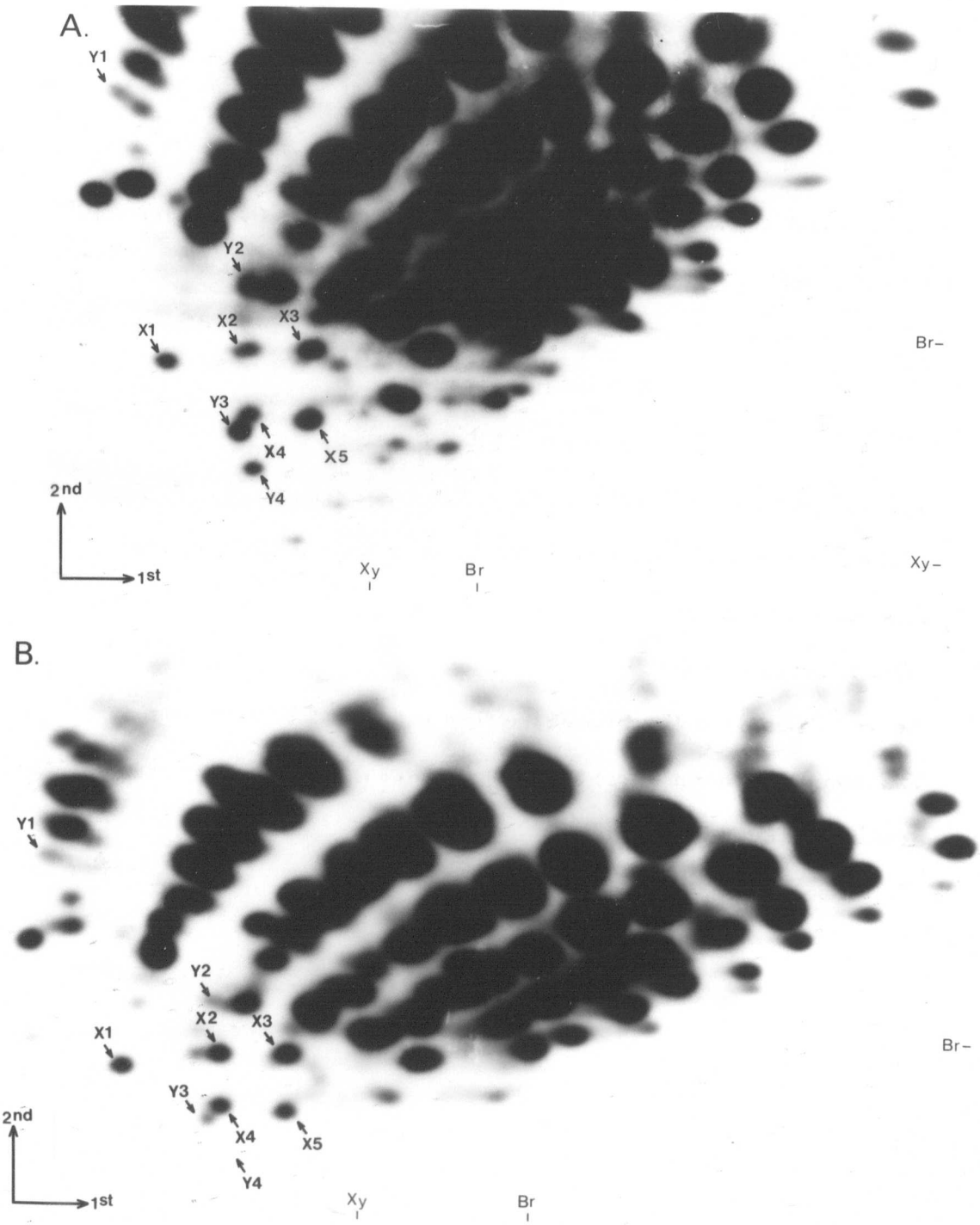
A. 8-14 B RNA. (An identical fingerprint is also presented in Figure 3.4 A).

B. Revertant R2 B RNA.

The oligonucleotides X1-X5 are labeled to allow orientation. The significance of the conserved oligonucleotides Y1-Y4 is outlined in the text. The oligonucleotide labelled Y4 is not seen in (B) but can be detected on the original autoradiogragh.

Arrows indicate the direction of the first and second electrophoresis.

The mobility of the markers bromophenol blue (Br) and Xylene cyanol (Xy) in each dimension is indicated.


A.
Y1
Y2
X1
X2
X3
Y3
X4
X5
Y4
2nd
1st
Xy
Br
Br-
Xy-
B.
Y1
Y2
X2
X3
X1
Br-
Y3
X4
X5
Y4
2nd
1st
Xy
Br

suggests that, using this technique, it would be very difficult to work out the position of the mutation(s) with in 8-14 B-RNA relevant to the ts phenotype.

3.4 Discussion.

Under permissive conditions the mutant 8-14 propagated sufficiently well in the systemic host to allow virus purification. Purified 8-14 preparations passaged at the permissive temperature contained approximately 5% virus which was phenotypically revertant as determined by ts assay. However, consistent with the work of Evans (1985b) virus isolated from a local lesion containing 8-14 was found to be completely ts by the same assay. This differance can be explained by considering that virus infecting the systemic host undergoes many more rounds of replication than virus infecting the local lesion host. Since the revertant has a selective advantage, the proportion of revertant virus is much greater in inocula derived from the systemic host.

In spite of the propensity of 8-14 to revert, the mutant was stably maintained by local lesion passage in "Pinto". On rare occasions (less than 3%) a local lesion was found to contain phenotypically revertant virus, and had presumably arisen as a result of reversion soon after infection.

Limited progress was made to further the phenotypic characterisation of 8-14. Evans (1985b) reported that some viral RNA was synthesised under the restricted conditions and it was intended to analyse this RNA. However, in contrast to the work of Evans (1985b), viral RNA could only be detected under permissive conditions. The reason for this is not certain, possibly a result of the different cultivar of cowpea used in this work. Alternatively, the hybridisation detected by Evans (1985b) and associated with RNA synthesis by 8-14 under restrictive conditions may not have been significantly above the background level. If this is the case, it might indicate that the phenotypic lesion is at the level of replication.

In vitro translation analysis of mutant 8-14 revealed that the ts defect on the B RNA did not affect expression of the 200K protein or the primary cleavage to give the 170K and 32K proteins. This indicates that the ts lesion in 8-14 does not affect translation or functioning of the 24K CPMV protease, at least for the first cleavage of the B RNA-encoded polyprotein into the 170K and 30K poducts.

Two-dimensional RNA fingerprinting was used to compare mutant, revertant and wild-type genomes. Comparisons are based on the principle that; after complete digestion with endonuclease T1; the large structurally unique oligonucleotides separate into patterns that are highly characteristic of the sequence from which they derive. Since characteristic oligonucleotides originate from all regions of the RNA molecule, the distribution of sequence similarities and differences may be surveyed over the entire viral genome.

To investigate the level of genetic alteration in 8-14, a comparison was made between wild-type and mutant genomes. The significance of the observed alterations was determined by comparing mutant and revertant genomes and by comparing the genomes of two wild-type CPMV isolates. The wild-type comparisons indicated the level of natural variation resolved by the fingerprinting technique and were included in the study since RNA genomes are known to evolve rapidly (Holland et al., 1982, van Vloten-Doting et al., 1985).

The comparison of viral genomes by RNA fingerprinting indicated that the nitrous acid treatment of 8-14 had not resulted in many genetic alterations. Indeed, mutant and wild-type genomes were more similar than those of two isolates of CPMV, CPMV.S1 and CPMV.S2, which showed sequence divergence as a result of evolution. Clearly, one might expect wild-type and 8-14 virus to have little evolutionary divergence since they have only recently been propagated as independent populations and this was found to be the case. This lack of variation is similar to that found in cloned U5-TMV after twenty passages in a systemic

host (Rodriguez-Cerezo and Garcia-Arenal, 1989).

Comparisons of wild-type and 8-14 B RNAs revealed specific changes. It was hoped that, by comparing mutant and revertant fingerprints the change(s) specific for the ts mutation would be revealed. However, mutant and revertant fingerprints were identical. There are two explanations for the inability to detect a genetic change relevant to the ts phenotype. Firstly, the genetic mutation relevant to the ts lesion might not be resolved by the fingerprinting technique. Secondly, one or more of the differences observed between the wild-type and 8-14 B RNAs are relevant to the ts phenotype but are maintained in a ts^- revertant of the mutant because the revertant contains additional secondary supressor mutations which are not resolved by the fingerprinting.

Genetic supression as a means of generating phenotypic revertants has been observed in animal virus systems (including reovirus, vesicular stomatitis virus, fowl plague virus and herpes simplex virus) but has not yet been documented in a plant virus. The mechanism of supressor reversion is uncertain. A possible explanation is that the supressor mutation produces compensatory alterations in another region of the protein or in a different protein that is in physical contact with the mutated protein, the compensatory alterations restoring a wild-type function under restrictive conditions, (Jarvik and Botstein, 1975, Morita et al., 1987). In the case of 8-14, since no altertions were resolved between revertant and mutant fingerprints the involvement of genetic supression remains uncertain.

Although a preparation of 8-14 could be purified from systemically infected leaves, the characterisation of 8-14 was hindered by the inability of the mutant to propagate well under permissive conditions and its propensity to revert. Sufficient 8-14 virus was purified to allow characterisation of in vitro translation and proteolysis under restrictive conditions. The results indicated that the ts lesion in 8-14 did not affect translation or the first cleavage of the

B RNA-encoded polyprotein catalysed by the 24K protease. The results of an investigation of RNA synthesis were not conclusive since problems were encountered when repeating the analysis of Evans (1985b). The further phenotypic characterisation of 8-14 was envisaged to be difficult, but of greater consequence were problems in characterisation of the genetic lesion. The fingerprinting technique was used and, although the results indicated that the use of nitrous acid as a mutagen had caused limited alterations to the sequence of the viral RNA, it was not possible to map the genetic alteration conferring the ts phenotype. This was a serious problem; alternative methods of genetic characterisation, such as cloning and sequencing of mutant and revertant genomes, being laborious and not practical. At this time, a full length clone of CPMV M RNA in the transcription vector pPM1 became available and it was thought that a study of directed mutants would be a more profitable way to increase our knowledge of CPMV. Thus the characterisation of 8-14 was not continued further.

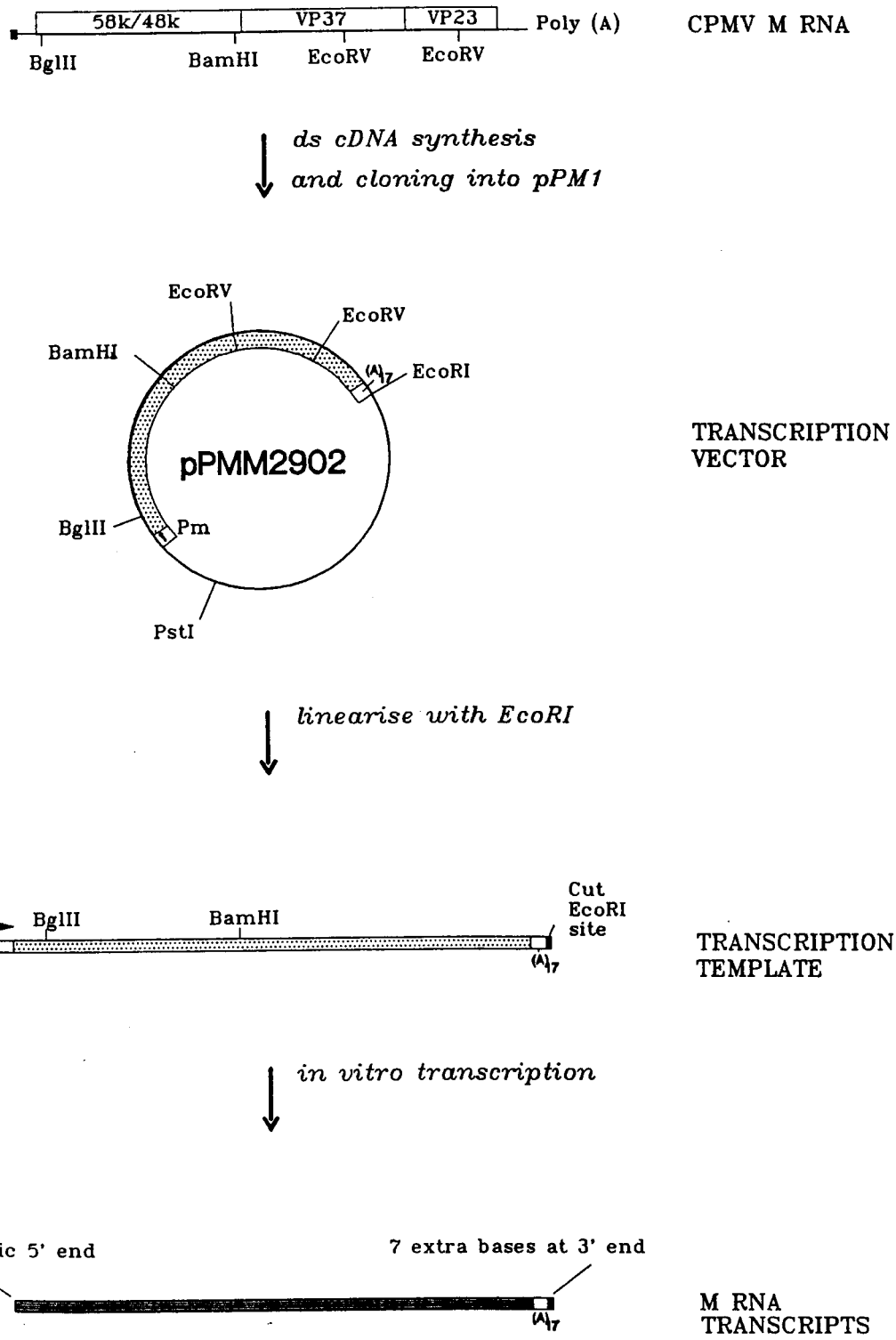


FIGURE 4.1.

pPMM2902 and in vitro transcription to generate M RNA transcripts.

The full-length cDNA of CPMV M RNA is cloned downstream of the Pm promoter in pPM1, giving pPMM2902. Several restriction enzyme sites are indicated to allow orientation. Transcription reactions are performed on pPMM2902 plasmids which have been linearised at the unique EcoRI site.

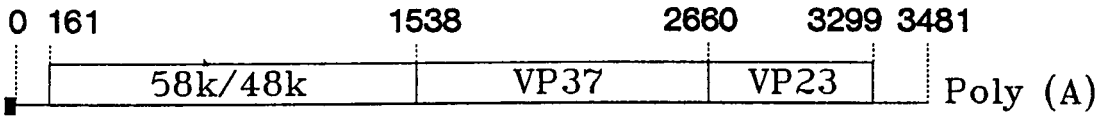
Chapter 4: Production and properties of *in vitro* transcripts generated from a full length cDNA clone of CPMV M RNA in pPM1.

4.1 Introduction.

The ability to produce biologically active transcripts from cDNA clones has been of great benefit in the study of plant viruses (see Section 1.3 B). The first full-length cDNA copies of CPMV M RNA were inserted into transcription vectors by Vos *et al.* (1984) who reported the construction of a clone containing a cDNA copy of M RNA downstream of the bacteriophage SP6 promoter. Transcripts from this clone gave the expected 105K and 95K *in vitro* translation products and deletion mutants of the clone were used to investigate M RNA expression and proteolytic processing *in vitro*. Subsequently, Verver *et al.* (1987) produced full length cDNA clones of B RNA in transcription vectors based on SP6 or T7 polymerase, *in vitro* transcription and translation of mutated cDNA copies allowing the identification of a B RNA-encoded protease (Verver *et al.*, 1987). These first cDNA clones generated transcripts with extra bases at their termini, the M RNA having 78 additional nucleotides at the 5' end and seven at the 3' end, and the B RNA having 35 extra bases at the 5' end and nine at the 3' end. These transcripts were not infectious, probably as a result of the many extra bases at their 5' termini.

In an attempt to circumvent the lack of infectivity of the CPMV transcripts described above, the construct pPMM2902 was made by Dr. G. P. Lomonossoff (for cloning strategy see Holness *et al.*, 1989). The construct pPMM2902 consists of a full-length cDNA clone of CPMV M RNA cloned downstream of the phage λ promoter in pPM1. *In vitro* transcription of pPMM2902 plasmids linearised at the unique *EcoRI* site after the viral cDNA insert, generates transcript RNAs which resemble CPMV M RNA (Figure 4.1). The transcripts are generated using *E.coli*

CPMV M RNA



Restriction map of full-length cDNA of CPMV M RNA

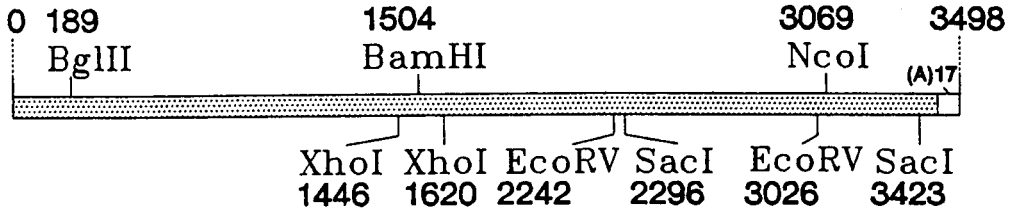


FIGURE 4.2.

A restriction map of a full-length cDNA copy of CPMV M RNA.

The restriction enzyme sites indicated on the M RNA cDNA do not occur within the pPM1 vector. The nucleotide position of each of the restriction sites is indicated.

A genomic map of CPMV M RNA is also shown to illustrate the positions of the restriction enzyme sites relative to the coding region of M RNA.

RNA polymerase and have authentic 5' termini. However, when compared with virion M RNA, transcripts from pPMM2902 lack the 5' VPg, have a shorter polyA tail of seventeen residues and have seven extra bases at their 3' termini. Figure 4.2 contains a restriction map of pPMM2902 indicating enzyme sites which are referred to in this thesis and their positions relative to the coding region of M RNA.

Preliminary investigations demonstrated that transcripts from pPMM2902 were translated in reticulocyte lysates to give the 105K and 95K proteins characteristic of CPMV M RNA and indicated that they were biologically active when electroporated in to cowpea protoplasts (Lomonossoff and Maule, unpublished data). These studies indicated that the pPMM2902/in vitro transcription system might represent a useful tool with which to study CPMV M RNA. The system potentially allows the generation of mutants at the DNA level by genetic engineering techniques, mutant transcripts then being analysed in vitro and in vivo.

The aim of the work presented in this chapter was to fully characterise the pPMM2902/in vitro transcription system prior to the use of pPMM2902 for reverse genetics. In order to generate the M RNA transcripts required for this optimisation, and to generate mutant transcripts in future studies, a plentiful supply of E.coli RNA polymerse was required. This chapter includes the purification of E.coli RNA polymerase of a quality sufficient to allow the production of biologically active transcripts from pPMM2902. The biological activity of transcripts was assessed by electroporation of protoplasts in the presence of virion B RNA. The preparation of B RNA of a purity sufficient for such experiments is described. In addition, the activity of transcripts and virion M RNA in vivo was compared and the amount of transcript required for effective in vivo analysis determined.

4.2 Purification of E.coli RNA polymerase.

E.coli RNA polymerase was purified as described in section 2.2(D). Figure 4.3 shows the protein profile of the polyethyleneimine precipitation and elution. E.coli RNA polymerase has four subunits, β, β', σ and α (with apparent molecular weights of 165,000, 155,000, 87,000 and 39,000 respectively). The doublet of high molecular weight polypeptides are the β and β' subunits of the polymerase and is used as a marker for the presence of the enzyme. All of the polymerase is precipitated from the cleared lysate by the addition polyethyleneimine to 0.35% (Figure 4.3, Tracks A and B). Washing the precipitate with buffer containing 0.5M NaCl removes 50-70% of extractable protein but none of the RNA polymerase (Figure 4.3, Track C) while washing with 1.0M NaCl buffer efficiently elutes the polymerase (Figure 4.3, Track D).

The column profile for the DNA-cellulose chromatography step is shown in Figure 4.5. The polymerase activity in eluted fractions was determined as described in the Materials and Methods and is shown in Figure 4.4 A. At early stages in the polymerase purification (including the DNA cellulose chromatography step) the assay for polymerase activity is adversely affected by impurities in the preparation and the activity values obtained serve only as a rough guide to the relative amount of activity present. Polymerase activity eluted in two peaks, but SDS/polyacrylamide gel analysis revealed that most of the enzyme (as judged by presence of the β,β' subunits, Figure 4.4 B) was eluted with the second peak of activity. Fractions corresponding to the second peak of activity were therefore combined and purified further.

The column profile for gel filtration chromatography is shown in figure 4.5. The peak of absorbance at fractions 19-23 was found to contain RNA polymerase activity. All subunits of the enzyme could be detected by SDS/polyacrylamide gel analysis of these fractions and there were few contaminant proteins, figure 4.5 B.

The material eluting in the void peak contains mostly lipid (Burgess and Jendrisak, 1975). The material eluting after the polymerase contains proteins which may affect the polymerase activity if they contaminate the final enzyme preparation.

Fractions 19, 20, 21 and 22 from the gel filtration colum were taken and individually dialysed against RNA polymerase storage buffer. The dialysates from fractions 19, 20 plus 21, and 22 were named preparation I, II and III respectively. All three final preparations contained enzyme activity. The purified enzyme is denoted JI E.coli polymerase.

Table 4.1 shows a summary of the JI polymerase purification. A yield of 4,400 units was purified from 30g of E.coli JM83. A comparison of the specific activity of the enzyme in preparation II with that in the cleared lysate reveals a 700 fold purification.

4.3 Production of in vitro transcripts.

Transcription reactions were performed on pPMM2902 transcripts which had been linearised at the unique EcoRI site occurring after the cDNA insert. All of the final preparations of JI E.coli polymerase directed the synthesis of transcripts from linearised pPMM2902. The largest transcript was of length equivalent to virion M RNA (Figure 4.6, Track B). Additional shorter products were also synthesised, a phenomenon also observed in transcription products synthesised by commercially available E.coli RNA polymerase preparations (Lomonossoff, personal communication). When in vitro transcription reactions were performed using between 0.1 and 10 units of JI polymerase per ug of linear DNA template, a concentration of 0.2-0.4 units/ug DNA template was found to be optimal. At concentrations below 0.2units/ug template the amount of transcripts synthesised declined dramatically and at concentrations above the optimum less full-length

FIGURE 4.3.

E.coli RNA polymerase purification: Protein profile of polyethyleneimine precipitation and elution.

10% polyacrylamide/SDS gel of samples of E.coli RNA polymerase at various initial stages of purification. Tracks were loaded with:-

A] Cleared lysate (30ug of total protein).

B] 0.35% polyethylimine supernatant (15ug).

C] 0.5M NaCl wash (7ug).

D] 1.0M NaCl eluate (3.5ug).

The positions of the β and β'subunits are indicated.

FIGURE 4.4.

DNA Cellulose Chromatograghy of E.coli RNA polymerase.

The ammonium sulphate precipitated 1M NaCl eluate was dissolved in TGED as described in 2.2 (D1) and loaded onto a 35ml DNA cellulose column at a flow rate of 25ml/hour. A 100ml salt gradient (0.5M-1.0M) was applied and eluted fractions of 2ml collected.

(A): A column profile of activity against eluted fractions is presented, 2ul aliquots of fractions were taken and assayed for enzyme activity as described in 2.2 (D2). Peak fractions as indicated (I—I) were pooled.

(B) Polyacrylamide/SDS gel analysis of eluted fractions. Aliquots (5ul) of eluted fractions were analysed on a 10% acrylamide gel as described in 2.2 D4. The numbers above the tracks indicate the fraction number, track (a) was loaded with an aliquot (20ug of total protein) of the sample loaded onto the DNA cellulose colum . The positions of the β and β' subunits are indicated. Peak fractions as indicated (I—I) were pooled.

FIGURE 4,3

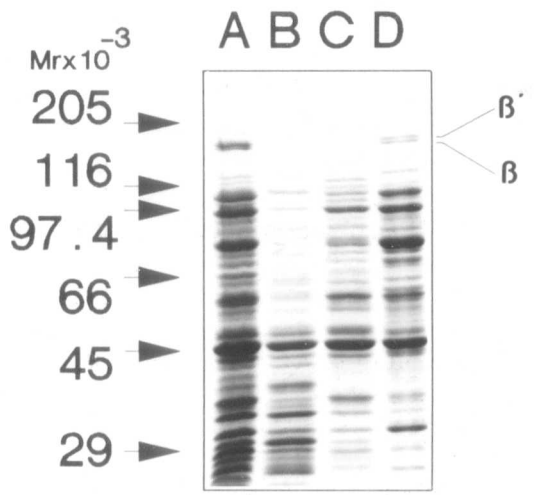
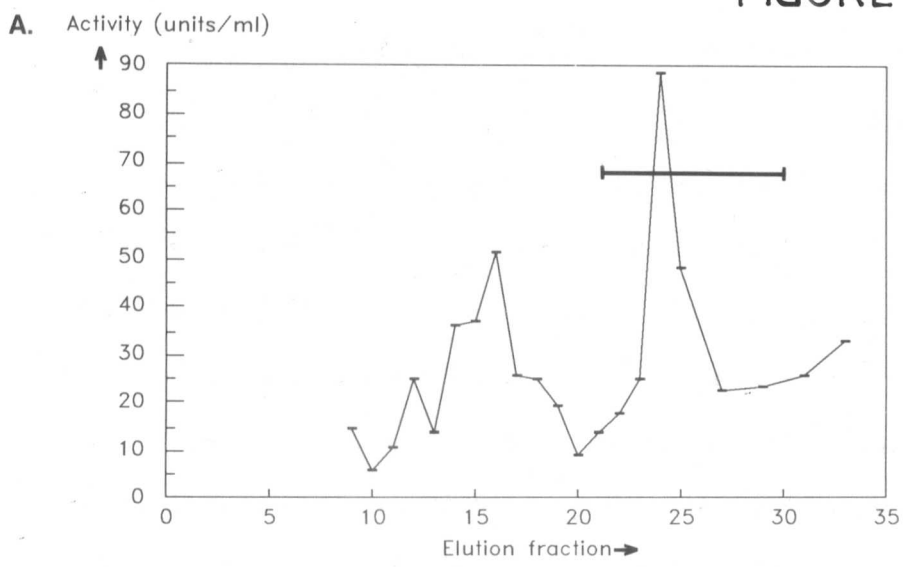


FIGURE 4,4



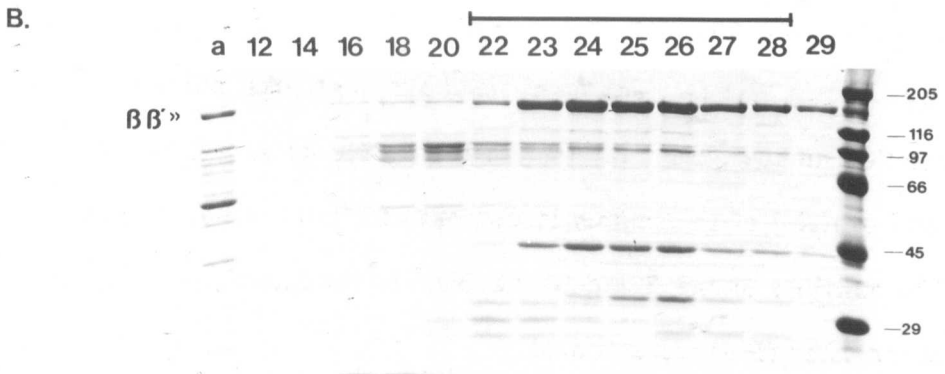


TABLE 4.1

Summary of E.coli RNA polymerase purification[a]

Stage of purification		Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (units)	Specific activity (units/ mg protein)	Fold purfication
Cleared lysate		440	2.95	1298	740	0.57	
PEI S/N		450	1.55	698			
0.5M Eluate		400	0.70	280			
1M Eluate		310	0.35	110			
Input DNA cellulose column		60	1.60	96	2481	25.80	45
Final polymerase preparations							
fractions	I[b]	0.5	1.50	0.75	197	262	460
	II[c]	0.8	9.45	7.56	3052	403	707
	III[d]	0.5	8.1	4.05	1123	277	486
Total[e]		1.8		12.36	4372	353	620

a From 30g cells.

b Dialysate from fraction 19 of gel filtration column.

c Dialysate from fractions 20 and 21 of gel filtration column.

d Dialysate from fraction 22 of gel filtration column.

e Data produced on combining fractions I, II and III.

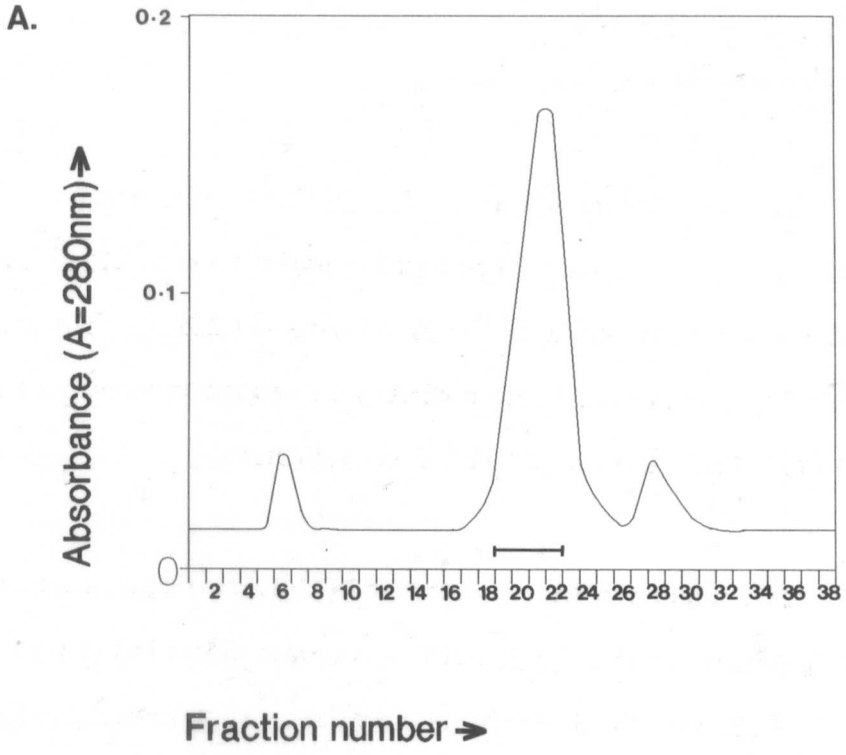
FIGURE 4.5.

Gel Filtration Chromatography of E.coli RNA polymerase.

The ammonium sulphate precipitated material from the DNA cellulose column was dissolved in buffer as described in 2.2(D1), and loaded on to a 50ml Superose 6 gel filtration column and eluted in the same buffer at 0.18ml/min. Fractions of 0.5ml were collected.

(A): A column profile of absorbance (A=280nm) against fraction number is presented. The E.coli RNA polymerase elutes in the peak at fractions 19-25. Fractions were taken as indicated (I—I) and dialysed against storage buffer.

(B): Polyacrylamide/SDS gel analysis of fractions eluted from the Superose 6 column. Aliquots (2.5ul) of eluted fractions were analysed on a 10% acrylamide gel as described in 2.2(D4). The number above each track indicates the fraction number. The positions of the polymerase subunits are indicated. The β and β' subunits have not been completely resolved and it is not known which of the two proteins migrating ahead of the 97.4K marker is the ω subunit.


A.
0·2
0·1
0
Absorbance (A=280nm)
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Fraction number
B.
18 19 20 21 22 23 24 25
ß ß'»
σ›
α›
205
116
97
66
45
29

transcript was produced, possibly as a result of inhibitors within the polymerase preparation. The use of 0.2-0.4units JI polymerase/ug linear DNA template generated 0.5-1.5ug of full length transcript/ug template, this activity being similar to that of commercially available polymerase.

The production of less than full-length transcripts from pPMM2902 is associated with the use of E.coli RNA polymerase since, when the M RNA cDNA is linked to an SP6 or T7 transcriptional promoter no additional transcripts are produced (Lomonssoff, personal communication). These shorter RNAs could arise by either transcription initiation at a site within the cDNA of pPMM2902 and termination at the site of linearisation, or by premature termination after correct initiation. Evidence that the largest, and most abundant, additional RNA species (Mwt approximately 7.0×10^5) arises from an alternative initiation site is provided by transcriptional analysis of pPMM2902 clones which have been linearised at sites within the cDNA insert (Figure 4.5). Linearisation of pPMM2902 with NcoI or EcoRV (Figure 4.2 shows the position of these enzyme sites within the M RNA cDNA) prior to in vitro transcription results in maximum length transcripts of 3000 nucleotides and 2200 nucleotides respectively. Shorter transcripts are again produced and, since they are consistently about 1000 nucleotides shorter than the maximum length transcripts, they must be produced by initiation at a fortuitous E.coli promoter sequence within the cDNA. By using the migration of transcripts of known size as standards for the gel system, the initiation site of the largest additional transcripts can be mapped on to the cDNA insert at a position between nucleotides 800 and 1200. However, computer searches revealed no significant E.coli promoter like sequences in this region of the cDNA. No attempt was made to separate the full-length from the shorter transcripts as the latter did not appear to interfere with the activity of the full-length transcripts (see Sections 4.4 and 4.6).

FIGURE 4.6.

In vitro transcription products generated from pPMM2902.

In vitro transcription products from pPMM2902 clones which had been linearised:-

(B) directly after the cDNA insert (by cutting with EcoRI).

(C) at position 3068 within the cDNA (by cutting with NcoI).

(D) at position 2211 within the cDNA (by cutting with EcoRV).

were electrophoresed on a 1% agarose gel containing formaldehyde. Virion RNA (1ug) was electrophoresed in track (A). The positions of M RNA and B RNA are indicated on the left hand side. The positions of the linear DNA templates are indicated by white arrows and the full length transcripts by white circles.

FIGURE 4.7.

In vitro translation of transcripts from pPMM2902.

Polyacrylamide/SDS gel analysis of in vitro translation products generated from M RNA and pPMM2902 transcripts. The positions of the 105K and 95K polyproteins and the β lactamase are indicated on the left hand side. The positions of molecular weight markers are indicated on the right hand side.

FIGURE 4,6

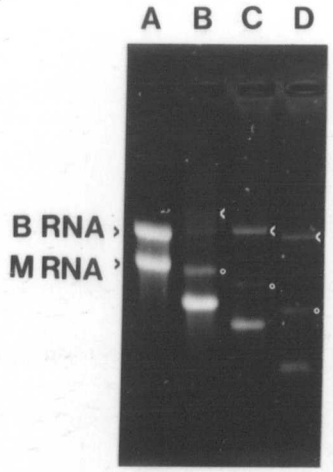
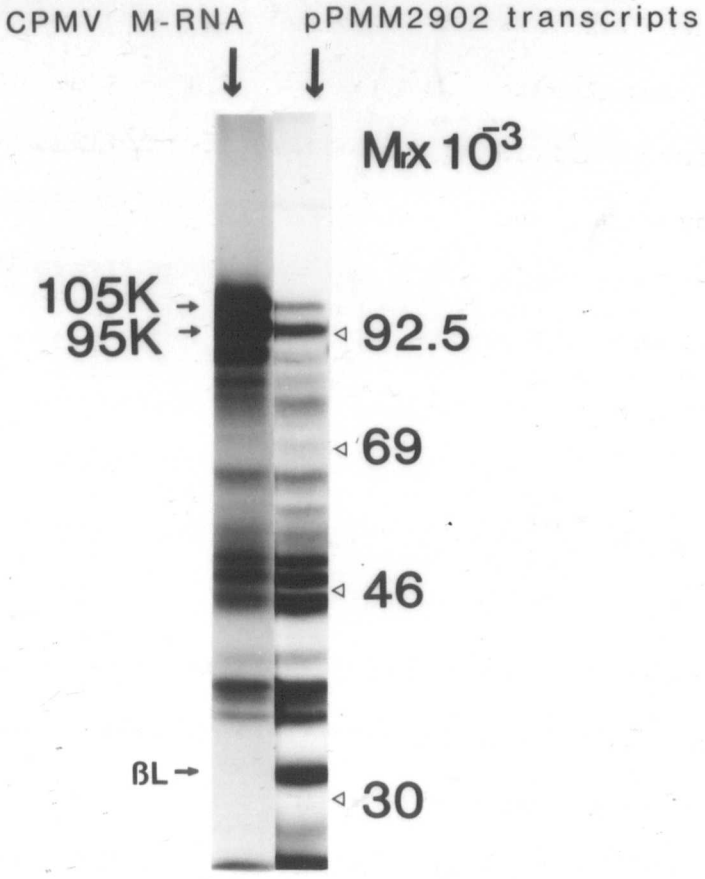


FIGURE 4,7



4.4 Production of in vitro translation products.

Before undertaking an in vivo analysis, the activity of transcripts generated from pPMM2902 using the JI polymerase was assessed in vitro. Translation of transcripts in reticulocyte lysates gave the 105K and 95K proteins characteristic of CPMV M RNA (Figure 4.7). The only significant difference between the products of virion M RNA and transcripts from pPMM2902 is the presence of the 30kDa β-lactamase in the latter. This is encoded by the ampicillin resistance gene of pPM1, mRNA from which is produced during the in vitro transcription reactions. The polypeptides of length less than 95K are thought to arise by premature termination and are a feature routinely observed upon in vitro translation of CPMV RNA.

4.5 Electroporation of protoplasts with CPMV virion RNAs and RNA from separated CPMV components.

Before assaying the biological activity of transcripts from pPMM2902 in vivo, it was necessary to determine the efficiency of protoplast infection by electroporation and to prepare virion B RNA free from contaminating M RNA.

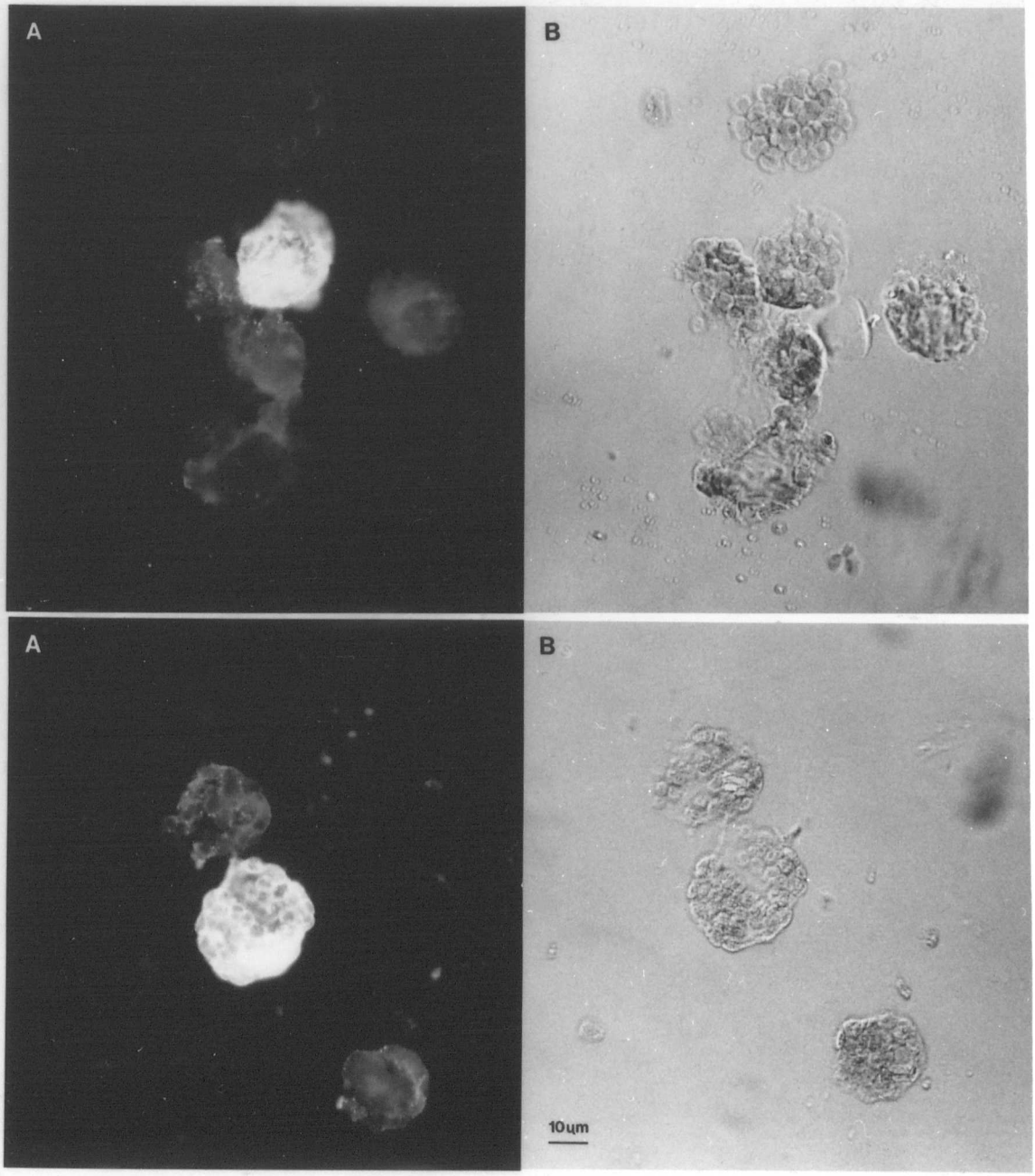
In order to determine the efficiency of infection after electroporation, various concentrations of a natural mixture of CPMV virion RNAs (between 1ng-2ug /10^6 protoplasts) were electroporated into cowpea protoplasts. At 72 hours post-electroporation the percentage of protoplasts producing viral coat protein was determined by immunofluorescent assay and used as a measure of infectivity. Figure 4.8 shows protoplasts 72 hours post inoculation. When viewed using epi-fluorescent optics, viral coat protein appears as intense fluorescence within the protoplasts localised around the chloroplasts. No fluoresence was observed in protoplasts mock inoculated.

From the dose/infection graph presented in Figure 4.9 it can be seen that the

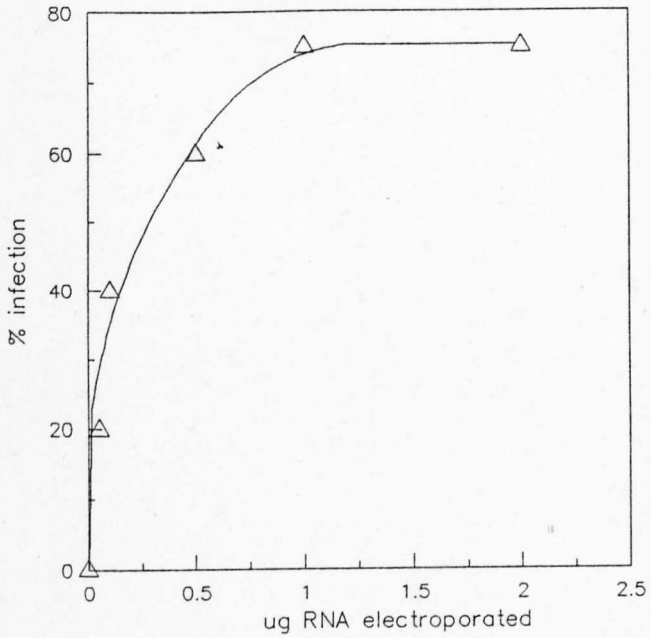
FIGURE 4.8.

Immunofluorescence of protoplasts.

Samples of protoplasts electroporated with CPMV RNA were examined and photographed using a Zeiss Universal Microscope equipped with epi-fluoresence (A) and Nomarski (differential interference contrast) optics (B).


A
B
A
B
10µm

Electroporation of Cowpea protoplasts with various concentrations of CPMV RNA.



CPMV RNA (ug)	% Infection
2	75
1	75
0.5	60
0.1	40
0.05	20
0.01	0
0.001	0

FIGURE 4.9.

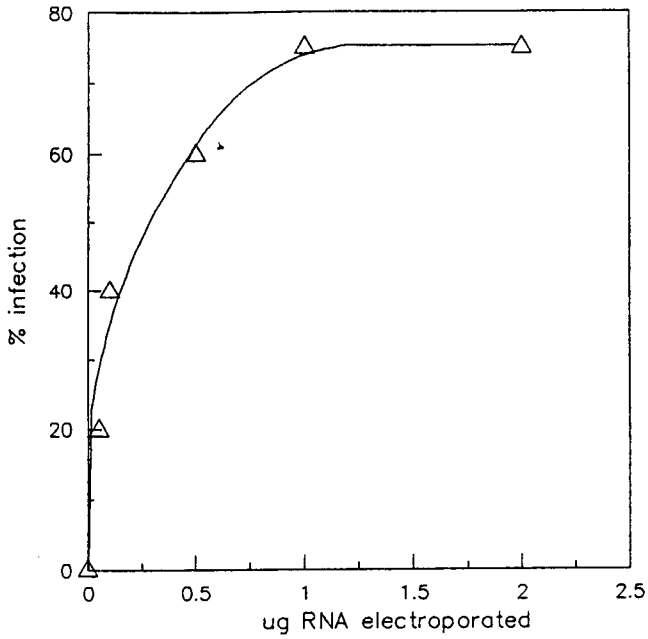
A dose/infection curve for the electroporation of CPMV virion RNA into cowpea protoplasts is presented. The level of infection was determined by immunofluorescent assay for antigen accumulation.

level of infection is a function of inoculum concentration. By interpolation, it can be estimated that 0.3ug of CPMV would give a 50% positive response. This value represents the dose of RNA required for 50% infectivity under the conditions of this experiment, the ID_{50}.

Since B RNA encodes all proteins necessary for replication of the CPMV genome, the biological activity of M RNA transcripts has to be assessed in the presence of virion B RNA. It was found that B RNA extracted directly from B components which had been separated from M components by Nycodenz gradient centrifugation, was not suitable for such experiments without further purification since it contained traces of M RNA. The B RNA was therefore further purified on sucrose gradients to eliminate contaminating virion M RNA. Typically 150ug of B RNA was applied to each 11ml sucrose gradient and yielded approximately 50ug of purified B RNA. When this highly purified B RNA was electroporated into protoplasts, a negative immunofluorescent assay was obtained (Table 4.2). The inability to detect the M RNA-encoded coat proteins by this assay indicates the absence of contaminating M RNA. The biological activity of this purified B RNA was confirmed by electroporating it in to protoplasts in the presence of virion M RNA (Table 4.2).

The immunofluorescent assay data from protoplasts electroporated with virion RNA or purified B RNA plus M RNA (Table 4.2) shows that the levels of infection varied between experiments. This variability was a reflection of the condition of the protoplasts. Although every effort was made to standardise the growth of plants and the procedure for protoplast isolation, batches of protoplasts isolated on different occasions differed in several ways. A good batch of protoplasts contained >90% spherical cells immediately after isolation, maintained a viability of >60% at 72 hours post electroporation and sustained a >65% infection (by immunofluorescent assay) after inoculation with 1ug CPMV RNA per

Electroporation of Cowpea protoplasts with various concentrations of CPMV RNA.



CPMV RNA (ug)	% Infection
2	75
1	75
0.5	60
0.1	40
0.05	20
0.01	0
0.001	0

FIGURE 4.9.

A dose/infection curve for the electroporation of CPMV virion RNA into cowpea protoplasts is presented. The level of infection was determined by immunofluorescent assay for antigen accumulation.

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TABLE 4.2 Electroporation of protoplasts with CPMV RNAs and immunofluorescent assay.

RNA electroporated per 10^6 protoplasts	N° independent experiments	% fluorescence with anti-capsid serum.
1ug CPMV RNA	>20	60-80
1ug M RNA + 1ug B RNA	>20	40-60
1ug B RNA	>20	0

TABLE 4.3 Electroporation of protoplasts with various concentrations of transcripts from pPMM2902 and immunofluorescent assay.

nucleic acid electroporated/10^6protoplasts			N° independent experiments	% fluorescence with anti-capsid serum
FLT[a]	LDT[b]	Total[c]		
[A].				
50	75	175-200	1	*
25	25	75-90	1	*
15	30	60-75	1	5
15	15	45-55	1	*
15	15	45-55	2	18-23
12	12	35-45	1	15
5-6	12-18	22-32	5	5-12
6	12	12-27	1	20
0.5-1	1-2	1.5-3	5	0-8
[B].				
0	100	100	1	0+
0	20	20	1	0
0	12	12	1	0
6	12	20-24	1	20
6	0	8-12	1	20

All electroporations carried out in the presence of 1ug B RNA / 10^6 protoplasts.

a Amount full-length pPMM2902 transcripts (ug) as estimated by ethidium bromide fluorescent quantitation.
b Amount linear DNA template (ug).
c Estimated amount of LDT plus FLT plus shorter transcripts plus B RNA.
* Value could not be determined accurately because of poor protoplast condition.
+ Although the condition of the protoplasts was poor, there was no immunofluorescence.

million protopasts. Batches of protoplasts that contained less than 70% spherical protoplasts immediately after isolation were discarded. The decreased infectivity of reconstituted CPMV RNAs, when compared with total RNA, may be explained by differences in the molar ratio of M RNA to B RNA and/or damage of the RNA during fractionation.

4.6 Electroporation of pPMM2902 transcripts into protoplasts: Assay of biological activity using immunofluorescence and Northern blotting.

When pPMM2902 transcripts generated using JI polymerase were electroporated into protoplasts in the presence of purified B RNA, viral antigen could be detected by immunofluorescent assay. Since the coat proteins are encoded by M RNA, this result provides evidence that the M RNA transcripts can at least be translated and probably replicated in the protoplasts. Table 4.3[A] shows immunofluorescent data from protoplasts electroporated with pPMM2902 transcription mixes which contained a range of concentrations of linear DNA template (LDT), full-length transcripts (FLT) and additional shorter transcripts.

The percentage of protoplasts which contained viral antigen was found to be dependent on the condition of the protoplasts, the amount of FLT and the amount of total nucleic acid electroporated. It was found that electroporation of protoplasts with greater than 45-55ug of total nucleic acid per million protoplasts caused protoplast clumping and loss of viability. Electroporation with 15ug of FLT gave antigen accumulation in approximately 20% of protoplasts - with one exception when protoplast viability was reduced. Electroporation with 6ug and 1ug of FLT gave infectivity values of approximately 12% and 4% respectively. It was decided to routinely electroporate a million protoplasts with 5-6ug of FLT as this gave antigen accumulation to a reasonable level (approximately 15% of the infection resultant from electroporation with M RNA plus B RNA) with no gross

reduction in protoplast viability and did not consume excessive amount of polymerase.

The data present in table 4.3(B) are from experiments designed to investigate the effect of the linear DNA template (LDT) in the electroporated transcription mixes. No antigen accumulates in protoplasts electroporated with LDT alone and infectivity of transcripts is not affected by the presence of the LDT. Therefore, LDT only displays biological activity through the transcripts which it directs in vitro, and presence of the LDT during electroporation has no adverse effects provided the total nucleic acid present is below 45-55ug/million protoplasts.

Confirmation that the transcripts generated using the JI polymerase could be replicated was provided by Northern blot analysis of the RNA produced in protoplasts. When protoplasts were electroporated with B RNA mixed with either viral M RNA or pPMM2902 transcripts, increasing amounts of M RNA could be detected in both cases with time post-electroporation (Figure 4.10, Tracks A and B). No M RNA specific sequences are detected in nucleic acid samples from protoplasts electroporated with B RNA alone, confirming the absence of contaminating M RNA (Figure 4.10, Track C). After electroporation with transcripts, RNA is detected somewhat later and does not accumulate to the same degree as when virion M RNA is used. These differences probably reflect the reduced infection levels achieved with M transcripts plus B RNA.

4.7 Analysis in whole plants.

In an attempt to demonstrate the biological activity of the transcripts from pPMM2902 at the whole plant level, samples of FLT (0.5ug-75ug) plus purified B RNA (250ng) were carefully inoculated onto a small area of a primary leaf on a cowpea plant. Initial experiments were hampered by traces of M RNA in the purified B RNA resulting in a systemic infection in cowpeas apparently inoculated

FIGURE 4.10

Northern blot analysis of M RNA produced in electroporated protoplasts. Nucleic acids were extracted from protoplasts at times after electroporation, in hours, indicated at the tops of the tracks. Each track was loaded with nucleic acids from 1.25 x 10^5 protoplasts.

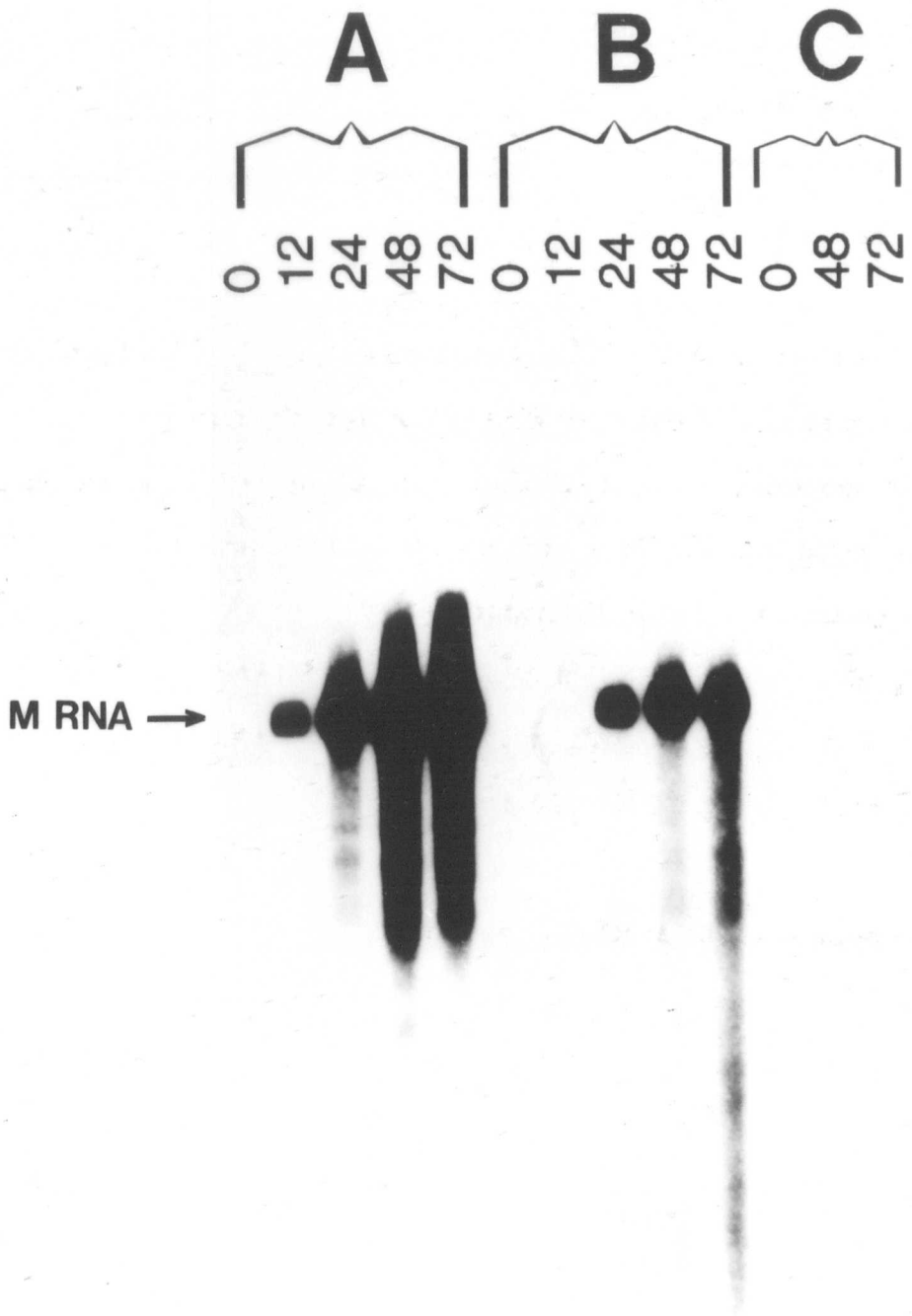
Protoplast samples were electroporated with:-

(A) 1ug B RNA plus 1ug virion M RNA/10^6protoplats.

(B) 1ug B RNA plus 6ug pPMM2902 transcripts/10^6protoplats.

(C) 1ug B RNA /10^6protoplats.

The position of the M RNA is indicated.


A
B
C
0 12 24 48 72
0 12 24 48 72
0 48 72
M RNA

with B RNA alone. The contamination of B RNA with M RNA presents much more of a problem when working at the whole plant level than in protoplasts since cell to cell spread allows amplification of traces of M RNA.

It was possible to obtain B RNA of a sufficient purity for plant experiments by using only the extreme fraction from the leading edge of B RNA after sucrose gradient centrifugation. The B RNA purified in this way was used in experiments to assess the biological activity of the transcripts. No symptoms were observed on plants inoculated with B RNA alone whilst addition of M RNA (even at concentrations as low as 50ng) resulted in a characteristic mosaic of the trifoliates and chlorosis of the inoculated leaf indicative of a CPMV infection. On plants inoculated with transcripts plus B RNA no symptoms developed and no CPMV RNA could be detected in the inoculated or trifoliate leaves.

4.8 Discussion.

A prerequisite of the use of the clone pPMM2902 as a source of mutants is the availability of large quantities of E.coli RNA polymerase. In this chapter I describe the production of 4370 units of RNA polymerase from 30g of exponential E.coli. This JI polymerase generated full length transcripts (0.5-1.5ug/ug template) at an optimal concentration of 0.2-0.4 units/ug template. This efficiency of full length transcript production compares well with that from pPM1 based clones of BMV (1-1.5ug/ug template, Ahlquist and Janda, 1984), TMV (250-340ng/ug template, Meshi et al., 1986), turnip crinkle virus (2.5ug/ug template, Heaton et al., 1989) and TRV RNA 1 (0.3ug/ug template, Hamilton et al., 1989).

In vitro transcription of pPMM2902 (with both commercially available and JI polymerases) produces shorter transcripts in addition to those of length equivalent to the cDNA. Extra transcripts are also generated from the cDNAs of BMV RNA 2, TMV and TRV RNA 1 when cloned into pPM1 (Ahlquist and Janda, 1984;

Dawson et al., 1986; Hamillton et al., 1989). No equivalent extra transcripts are reported when cDNA clones of cowpea mosaic virus (Vos et al., 1988b) and BMV (Janda et al, 1987) are transcribed using T7 polymerase, reflecting the higher specificity of T7 polymerase for its promoter sequence. Consistent with this, the largest additional transcript does appear to be synthesised from pPMM2902 by internal initiation at a non-authentic promoter site. The shorter length transcripts from pPM2902 encode no detectable protein in vitro and are not replicated in cowpea protoplasts. The ratio of full-length to shorter length products synthesised from pPMM2902 varied between different batches of commercial enzyme, a similar variability of enzyme functioning being observed by others (Janda et al., 1987, Hamilton et al., 1989). Thus an additional advantage of purifying a large quantity of JI polymerase was consistency of transcript production.

In order to determine the biological activity of pPMM2902 transcripts generated using JI-polymerase, protoplasts were electroporated with transcripts in the presence of virion B RNA. Such experiments were possible since the B RNA could be purified to remove traces of contaminating virion M RNA. Electroporation of protoplasts was found to be highly efficient, giving an ID50 of 0.3ug for CPMV RNA. By comparison, polyethylene glycol inoculation of protoplasts with 100ug of CPMV RNA was shown by De Varennes (PhD thesis) to give only a 35% infection level using an equivalent number of protoplasts and an identical immunofluorescent assay. Although electroporation is relatively efficient compared with other methods of inoculation, an ID50 of 0.3ug per 10^6 protoplasts still represents $5x10^4$ RNA molecules per protoplast, illustrating the general inefficiency of the infection process.

Transcripts generated from pPMM2902 using JI polymerase are biologically active when electroporated into protoplasts, replicating and producing coat protein. The infectivity of transcripts was found to be independent of the

presence of linear DNA templates unless the total nucleic acid concentration exceeded 45-55ug/10^6 electroporated protoplasts, the quality of protoplasts being adversely affected under these conditions. A concentration of 6ug full length transcript/10^6 protoplasts gave a good infection after electroporation and it was decided to use these conditions for future experiments.

Transcripts from pPMM2902 lack the VPg and are still biologically active in protoplasts. This observation is consistent with other data showing that the VPg is not needed for infectivity of CPMV (Stanley et al., 1978; Vos et al., 1988b). However, transcripts generated from pPMM2902 are an order of magnitude less infectious than virion M RNA and a possible reason for this is instability owing to lack of a VPg. The infectivity of pPMM2902 transcripts might be increased by capping (presence of 5' m^7Gppp) as this process increases the infectivity of transcripts of BMV (Ahlquist et al., 1984), alfalfa mosaic virus RNA 4 (Loesh-Fries et al., 1985), TMV (Dawson et al., 1986, Meshi et al., 1986) and TCV (Heaton et al., 1989). Presumably the cap affords some protection to the 5' end of each transcript and so enhances infectivity. Unfortunately, transcripts from pPMM2902 could not be capped as the required m^7GpppU is not commercially available. Instability of pPMM2902 transcripts, owing to the lack of any protective structure at their 5' termini, may be associated their inability to propagate in plants when inoculated with B RNA. Indeed, transcripts from barley stripe mosaic virus (Petty et al., 1989) and turnip yellow mosaic virus (Domier et al., 1989) and not infectious unless they are capped.

Recently, Vos et al. (1988b) have produced clones containing full-length cDNA copies of both M and B RNA downstream of a modified T7 promoter. The RNA generated from these clones does appear to be infectious in cowpea protoplasts though at a low level, only 0.05% of protoplasts becoming infected when inoculated with a mixture of the M and B transcripts (Vos et al., 1988b). In

contrast, transcripts from pPMM2902 give a comparatively high level of infection when coinoculated with virion B RNA.

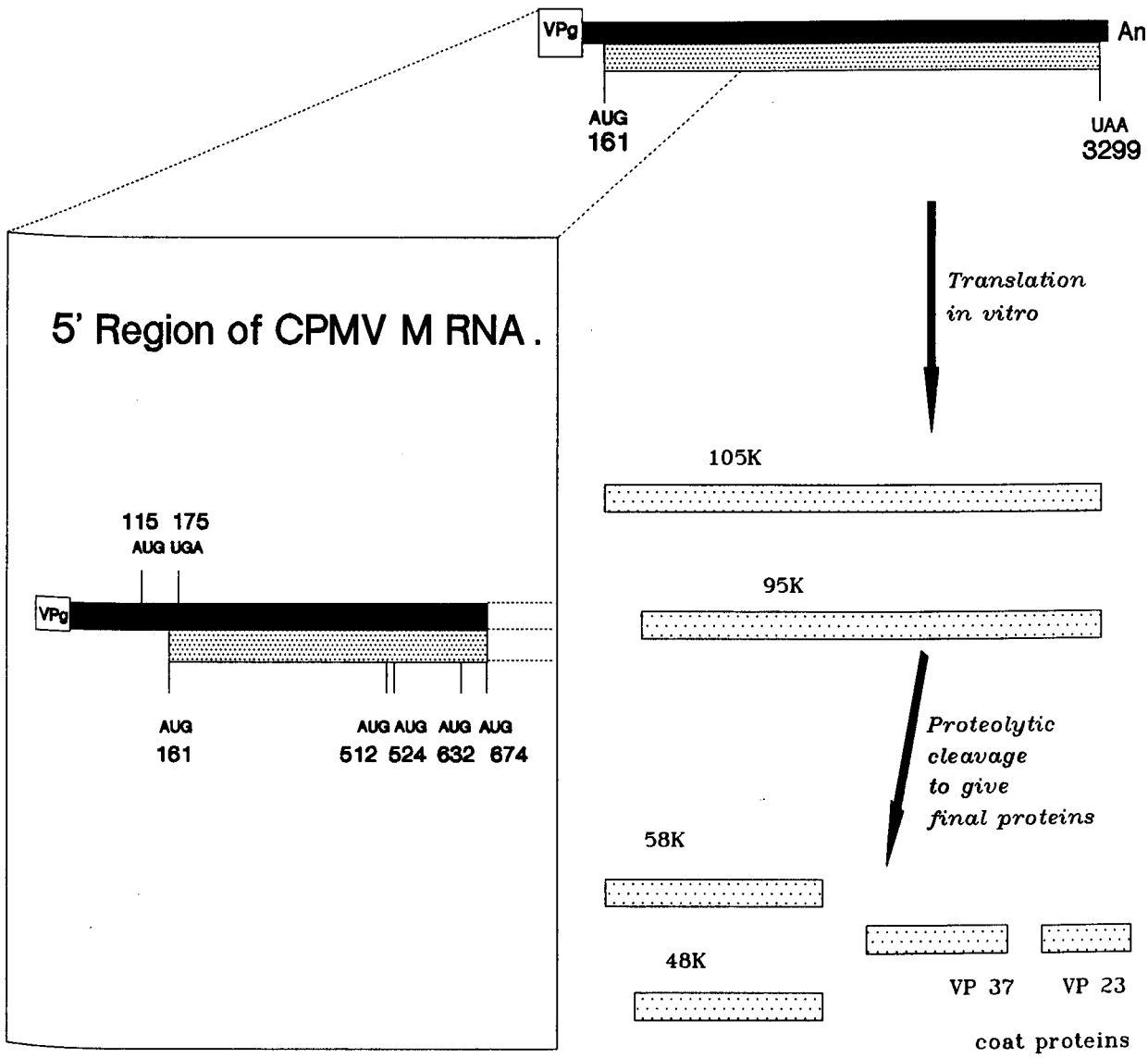
Overall, the results show that that the pPMM2902/transcription system is suitable for the further analysis of CPMV M RNA by reverse genetics.

Chapter 5. Identification of the initiation codons for translation of cowpea mosaic virus M RNA.

5.1 Introduction.

Both in vitro and in vivo translation studies have enabled models for the expression of CPMV B RNA and M RNA to be proposed (see Chapter 1 and Figures 1.2 and 1.3). A detailed picture of the expression of B RNA has been obtained (Wellink et al. 1986), but less is known about the expression of M RNA, particularly in vivo. The possible initiation codons for translation of the 105K and 95K proteins are shown in figure 5.1.

The proposed use of the first in-phase AUG for the synthesis of the 105K protein (van Wezenbeek et al., 1983) is supported by data obtained by translating transcripts from a full-length cDNA clone of CPMV M RMA (pSPM5) in which a frame-shift was introduced downstream of position 161 (Vos et al., 1984). In this piece of work, mutant pSM5Δ*Bgl*II was produced by cutting pSPM5 at its unique BglII (position 189 of the M RNA sequence) and filling-in the protruding ends of the restriction site before religation. The addition of four nucleotides in this mutant results in the frame-shift, a translational start at the AUG codon at position 161 being expected to terminate twenty-two codons downstream at a UAA stop codon. Transcripts, generated from pSM5Δ*Bgl*II in vitro using SP6 polymerase, directed the synthesis of the 95K protein but not the 105K protein in rabbit reticulocyte lysates, demonstrating that the two proteins are synthesized independently in vitro. However, since neither the 105K or 95K protein has yet been detected in plants or protoplasts infected with CPMV, the relevance of the in vitro data to the in vivo situation remained unclear. Transcripts from pSM5Δ*Bgl*II could not be analysed in vivo as they were not derived from an infectious cDNA clone.


CPMV M RNA
VPg
An
AUG
161
UAA
3299
Translation
in vitro
5' Region of CPMV M RNA.
115 175
AUG UGA
VPg
AUG
161
AUG AUG AUG AUG
512 524 632 674
105K
95K
Proteolytic
cleavage
to give
final proteins
58K
48K
VP 37
VP 23
coat proteins

Two AUG codons (at positions 512 and 524) have previously been suggested as the initiation sites of synthesis of the 95K protein (van Wezenbeek et al., 1983) with initiation at the AUG at position 524 being the recently favoured option (Wellink et al., 1987a).

To identify the initiation codons used for translation of CPMV M RNA directed mutants of pPMM2902 were produced. The in vitro generated transcripts were analysed in reticulocyte lysates and in protoplasts. Initially, a frame shift mutant, similar to that of pSMΔ*Bgl*II, was produced in pPMM2902 and in vitro generated transcripts studied in vitro and in vivo.

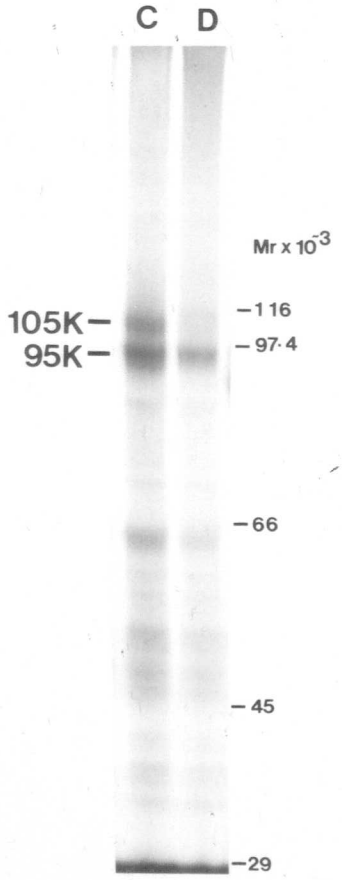
A further series of mutants was produced having alterations in AUG codons thought to be functional in initiation of translation. Mutant transcripts were translated in reticuloyte lysates and electroporated in to cowpea protoplasts.

5.2 Production and analysis of a frame-shift mutant, pIM1.

Mutant pIM1 was produced by cutting pPMM2902 at the unique BglII site at position 189 of the M RNA-specific region and filling-in the ends of the restriction site before religation. This is predicted to insert four base pairs and create a unique ClaI site at the site of the insertion. The mutation was confirmed by restricting pIM1 with ClaI. The ClaI (ATCGAT) site in pIM1 is preceeded by a guanosine. This is significant as the adenine in the sequence GATC is subject to methylation by host encoded dam methylase (Hattman et al., 1978) and ClaI is methylation sensitive. In order to restrict pIM1 with ClaI it was therefore necessary to propagate the plasmid in the dam$^-$ host GM242.

In contrast to the expression of pPMM2902 transcripts in reticulocyte lysates, transcripts generated in vitro from pIM1 directed the synthesis of the 95K protein and not the 105K protein, Figure 5.2 A. This is consistent with the predicted effect of the frame shift mutation on the reading frame proceeding the

A.


C
D
Mr x 10-3
105K
95K
116
97·4
66
45
29

B.


E
F
G
0 24 48 72 0 24 48 72 0 24 48 72
M RNA

AUG at 161, initiation at this AUG no longer directing the synthesis of a protein of apparent molecular weight 105kDa but a protein of only twenty-two amino acids.

Transcripts from pIM1 and pPMM2902 were electroporated into protoplasts in the presence of purified B RNA. The biological activity of transcripts from pPMM2902 was confirmed by immunofluorescent staining of the protoplasts with anti-capsid antiserum and by Northern blot analysis of the progeny RNA. However, no capsid protein was detected in protoplasts electroporated with pIM1 transcripts and no progeny M RNA detected by Northern blotting (Figure 5.2 B). In the nucleic acid samples from protoplasts electroporated with pIM1 transcripts, the smear of lower molecular weight hybridising material is due to the presence of degraded DNA template and not progeny RNA molecules. The results indicate that transcripts from pIM1 replicate extremely poorly or not at all.

To investigate the expression of the 105K protein further and to determine the site of initiation of the 95K protein, site-directed mutations were produced in the M RNA cDNA.

5.3 Production of site-directed mutants.

Four site-directed mutants of the wild-type cDNA clone (pPMM2902) were produced. Details of the mutations are presented in Table 5.1. Each of the mutants pGM161, pGM512 and pGM524 has one mutated AUG codon at position 161, 512 or 524 respectively; pGM512+524 is a double mutant with changes in the AUG codons at positions 512 and 524. The mutants were produced by cutting pPMM2902 at two unique sites: the BamHI site at position 1504 within the M RNA cDNA and the PstI site occuring 0.9Kb before the cDNA (see Figure 4.2 for a restriction map of the M RNA-specific region of pPMM2902). The 2.4Kb PstI-BamHI fragment, containing the 5' 1.5Kb of the M RNA cDNA, was subcloned into PstI-BamHI cut

TABLE 5.1

SITE DIRECTED MUTANTS PRODUCED.

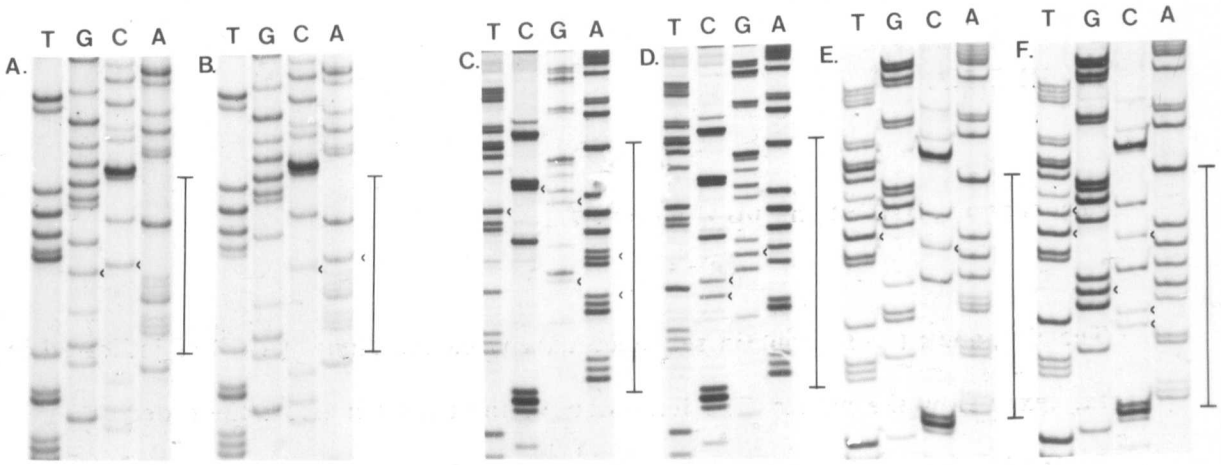
Name of clone.	Nucleotides around the ATG at position 161	512	524	Changes from the wild-type.
pPMM2902	ACAATGTTT	GAAATGGAA	ATTATGAGC	Wild-type.
pGM161	ACAAGCTTT ▲▲	Wild type.	Wild-type.	AGC (ser codon) at 161. HindIII site at '160.
pGM512	Wild-type.	GAACTCGAG ▲ ▲ △	Wild-type.	CTC (leu codon) at 512. XhoI site at 512.
pGM524	Wild-type.	Wild-type.	ATTACTAGT ▲▲ △	ACT (thr codon) at 524. SpeI site at 524.
pGM512+524	Wild-type.	GAACTCGAG ▲ ▲ △	ATTACTAGT ▲▲ △	As pGM512 & pGM524.

▲ Indicates nucleotide changes from the wild-type.

△ Indicates nucleotide changes which have no effect at the amino acid level.

M13 mp19 replicative form DNA and site directed mutagenesis performed as described in Materials and Methods. Three different oligonucleotides:
"oligo 161" d(GAAAGAAAAGCTTGTACTGG),
"oligo 512" d(CATAATGCTCTCGAGTTCAAATTTG) and
"oligo 524" d(GGAATACCACGACTAGTAATGC) were used to create three independent mutants. The oligonucleotides were designed to mutate the M RNA-specific DNA at the ATG codons corresponding, respectively, to positions 161, 512 and 524 of the M RNA sequence. A fourth mutant was produced by taking recombinant M13 mutagenised with "oligo 512" and subsequently mutagenising it with "oligo 524". The base changes made were selected to create a new restriction site to facilitate screening for the desired mutation. M13 clones having the mutations were identified by restriction enzyme digestion of mini preparations of their replicative-form DNAs.

The nucleotide alterations were confirmed by sub-cloning and sequencing across the mutated region (Figure 5.3). In order to sequence the mutated AUG codons at positions 512 and 524, replicative form M13 mp19 clones were cut with HindIII and BamHI and the 1kb fragment corresponding to sequences 482-1504 of M RNA was cloned into HindIII/BamHI cut Bluescript M13-. After rescue with helper phage, the Bluescript inserts were then sequenced from the HindIII site corresponding to position 482 of the M RNA and through the AUG codons at positions 512 and 524. In order to sequence the mutated AUG codon at position 161, replicative form M13 clones were cut with PstI and BglII; the BglII site occuring at position 189 of the M RNA cDNA. The 1.9Kb fragment was cloned into PstI/BamHI cut Bluescript M13+, the BglII sticky end of the insert being compatible with the BamHI sticky end of the Bluescript vector. The insert from the single-stranded DNA was then sequenced from the BamHI site, corresponding to the complementary strand of the M RNA cDNA from the BglII site at 189 and



A. pGM161

173 Hind III 151

TGAAAGAAAAGCTTGTACTGGTG

B. pPMM2902

173 161 151

GTA

TGAAAGAAAACATTGTACTGGTG

C. pPMM2902

502 512 524 536

CAAATTTGAAATGGAAAGCATTATGAGCCGTGGTA

D. pGM512

502 Xho I 524 536

CAAATTTGAACTCGAGAGCATTATGAGCCGTGGTA

E. pGM524

502 512 Spe I 536

CAAATTTGAAATGGAAAGCATTACTAGTCGTGGTA

F. pGM512+524

502 Xho I Spe I 536

CAAATTTGAACTCGAGAGCATTACTAGTCGTGGTA

continuing through the AUG at position 161. Sequence analysis confirmed the site-directed mutations and indicated that there were no additional base changes in the region of the mutations.

To produce the pGM mutants, the altered PstI-BamHI fragments in M13 mp19 were excised and cloned back into the vector fragment of PstI-BamHI cut pPMM2902. Each of the mutants pGM161, pGM512 and pGM524 has one mutated AUG at position 161, 512 or 524 respectively; pGM512+524 is a double mutant with changes in the AUG codons at positions 512 and 524.

5.4 In vitro expression of site-directed mutant transcripts.

When transcripts from EcoR1-linearised pPMM2902 and the pGM series of mutant derivatives were translated in rabbit reticulocyte lysates, the results were as shown in Figure 5.3. While transcripts from pPMM2902 gave both the 105K and 95K proteins, transcripts from pGM161 gave only the 95K protein (Figure 5.3, track C). This unambiguously identifies the AUG at 161 as the initiator for the synthesis of the 105K protein, at least in vitro. The fact that pGM161 transcripts still produced the 95K protein confirms that this product has an independent initiation site and does not arise through processing of the 105K protein.

Transcripts from the single mutants pGM512 and pGM524 both directed the synthesis of the 105K protein (Figure 5.3, tracks D and E). More surprisingly, both mutants also synthesised the 95K protein, this synthesis only being abolished in the case of transcripts from the double mutant, pGM512+524 (Fig 5.3, track F). These results indicate that the 95K protein synthesis can initiate in vitro from either of the AUG codons at positions 512 and 524. However, the "95K" protein directed by transcripts from pGM512 appears to migrate slightly ahead of that produced from pPMM2902 and pGM524 transcripts (Figure 5.3, compare track D

FIGURE 5.4.

In vitro translation products directed by transcripts from the pGM mutants.

Products from translation of were analysed on a 10% polyacrylamide/SDS gel. The tracks were loaded with the products of the translation of:-

(A) virion M RNA.

(B) transcripts from pPMM2902.

(C) transcripts from pGM161.

(D) transcripts from pGM512.

(E) transcripts from pGM524.

(F) transcripts from pGM512+524.

The size of protein markers used is indicated on the right hand side. The positions of the 105K and 95K primary translation products are marked on the left hand side. The band indicated by ßL is believed to be ß-lactamase.

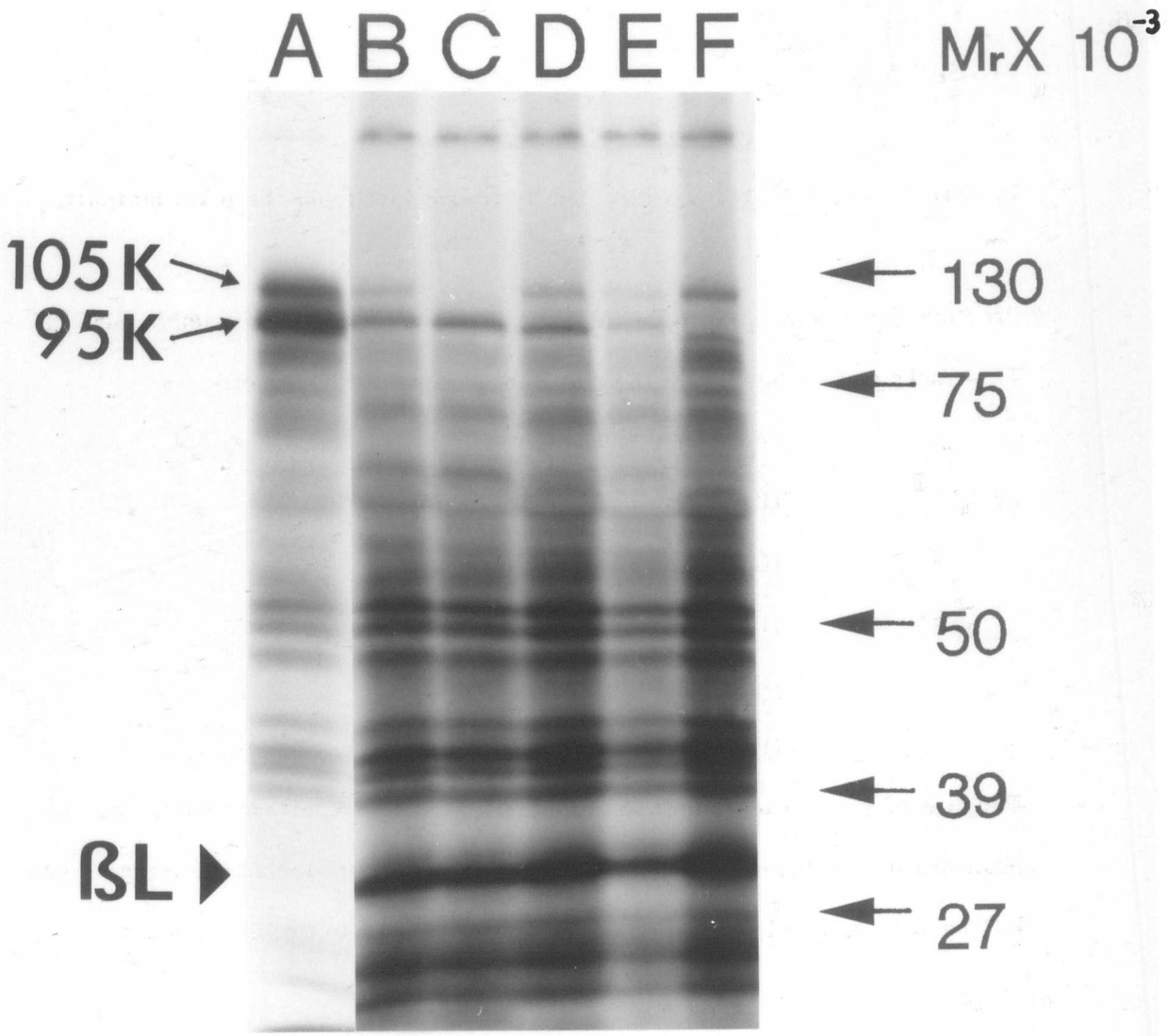

A B C D E F
MrX 10-3
105K
95K
ßL
130
75
50
39
27

TABLE 5.2

IMMUNOFLUORESCENT ASSAY OF PROTOPLASTS INOCULATED WITH VIRAL M-RNA OR TRANSCRIPTS.

RNA[a]	ug RNA per 10^6 protoplasts	% fluorescent cells with anti-capsid serum. Experiment A	Experiment B
virion M-RNA	1	35	50
pPMM2902 transcripts	6	12	20
pGM512 transcripts	6	12	b
pGM524 transcripts	6	9	b
pGM512+524 transcripts	6	9	b
pGM161 transcripts	6	b	0
" "	12	b	0
virion B-RNA	1	0	0

a The first column indicates the kind of RNA used to inoculate the protoplasts, all RNA was inoculated in the presence of B-RNA (1ug/10^6 protoplasts).

b Not included in this experiment.

with tracks B, C and E). This suggests that the AUG at 512 is probably the one actually used on wild-type RNA.

5.5 In vivo analysis of site-directed mutant transcripts.

To analyse the properties of the mutants in vivo, transcripts from pPMM2902, pGM161, pGM512, pGM524 or pGM512+524 were electroporated into cowpea protoplasts in the presence of virion B RNA. The biological activity of the transcripts was assessed by immunofluorescent staining of the protoplasts with anti-capsid antiserum and by Northern blot analysis of the progeny RNA.

The immunofluorescence data presented in Table 5.2 indicate that all the constructs except pGM161 produced biologically active transcripts. Though the level of infection varied slightly between experiments, transcripts from pGM512, pGM524 and pGM512+524 consistently gave infection levels comparable to the wild-type (pPMM2902) transcripts. Coat protein antigen was never detected in protoplasts electroporated with virion B RNA alone. The fact that transcripts from the double mutant, pGM512+524, produced coat protein antigen indicates that synthesis of the 95K protein is not required for coat protein production in vivo.

Northern blot analysis of RNA extracted from protoplasts at various times after electroporation showed that transcripts from all the mutants except pGM161 can be replicated in the presence of B RNA (Figure 5.5). The smear of hybridising material in the zero time points from samples of protoplasts electroporated with transcripts is due to the presence of degraded DNA template. Transcripts from pGM161 consistently failed to give any detectable signal even when the amount of input transcript was doubled. The rate of M RNA accumulation in protoplasts electroporated with transcripts from mutants pGM512, pGM524 and pGM512+524 was similar to that which occurred when wild-type transcript was used. The minor variations in the apparent rates of RNA accumulation of the mutants are probably

FIGURE 5.5.

Northern blot analysis of CPMV M RNA produced in protoplasts electroporated with the pGM mutant transcripts.

Nucleic acids were extracted from protoplasts at times after electroporation, in hours, indicated at the tops of the tracks. Each track was loaded with nucleic acids from 1.25 x 10^5 protoplasts. Protoplasts were electroporated with either:-

(b) B RNA (1ug/10^6 protoplasts),

or B RNA (1ug/10^6 protoplasts) plus transcripts (6ug/10^6 protoplasts) from:-

(a) pPMM2902.

(c) pGM512.

(d) pGM524.

(e) pGM512+524.

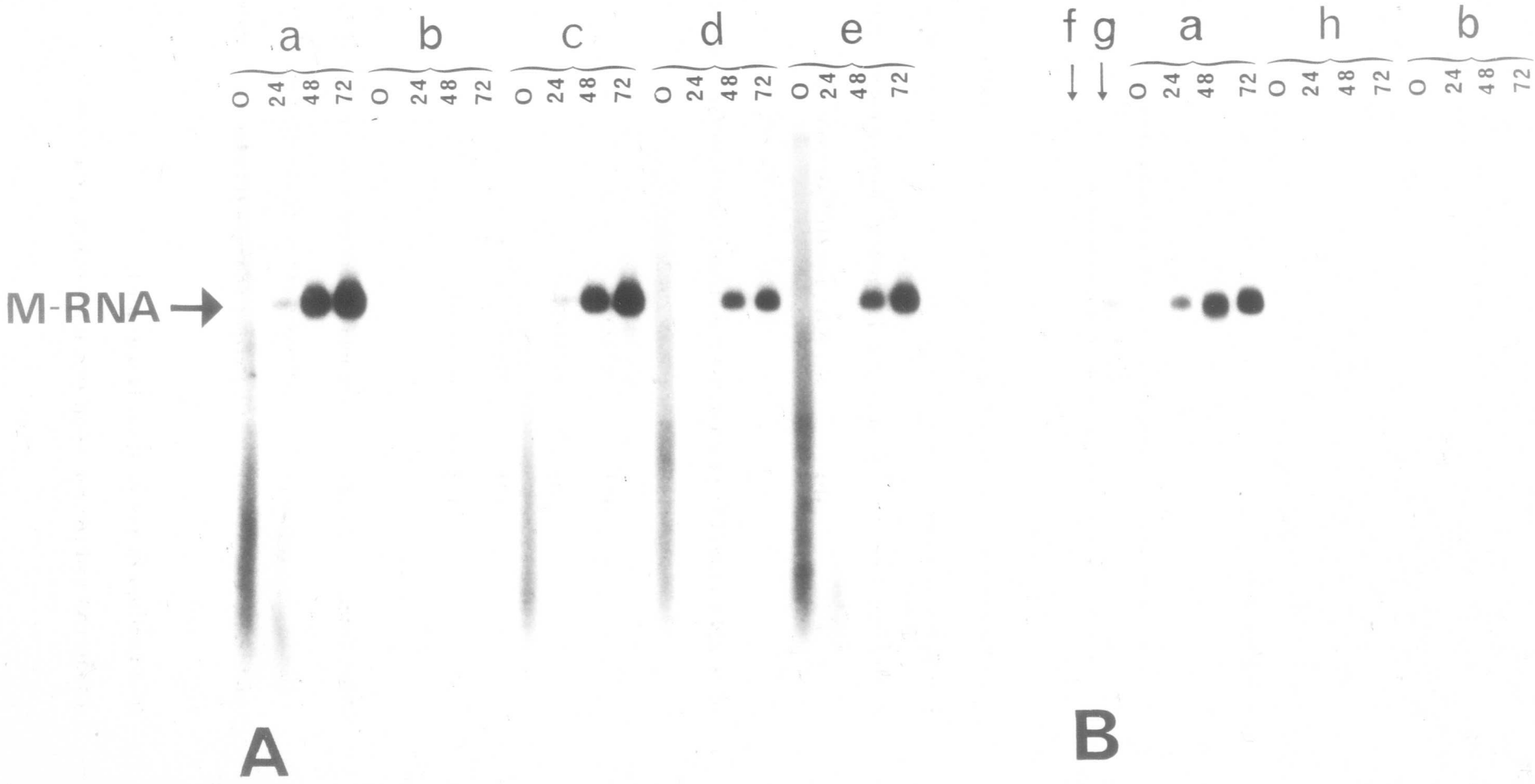
(h) pGM161.

Also, track (f) contains nucleic acids from protoplasts electroporated in the absence of transcripts.

And track (g) Extract as track (f) plus 8ng CPMV RNA.

The position of the M RNA is indicated.

Panels (A) and (B) represent two independent experiments.


a
b
c
d
e
f
g
a
h
b
0
24
48
72
M-RNA
A
B

not significant. The accumulation of viral RNA to the levels seen in Figure 5.5 suggests that the progeny RNA molecules are encapsidated since unencapsidated RNA is unstable and does not accumulate to a significant extent (de Varennes and Maule, 1985). These results demonstrate that production of the 95K protein is not necessary for M RNA replication or encapsidation in protoplasts.

5.6 Detection of the 48K and 58K proteins in protoplasts.

When samples of protoplasts infected with a mixture of B RNA and either virion M RNA or pPMM2902 transcripts were assayed for the presence of the 48K and 58K proteins by Western blotting using the 58/48K antiserum, both proteins could be detected in the S30 fraction (Figure 5.6, lanes A and B). This result contrasts with a previous report (Wellink et al., 1987a) where only the 48K was detected and is the first time the 58K protein has been identified in vivo. As the 58K protein is thought to be derived from the 105K protein (see Figure 1), the detection of the 58K protein provides direct evidence for the existence of the 105K protein in vivo . Significant amounts of the 48K but not the 58K protein were also found in the P30 fraction in agreement with the results of Wellink et al. (1987); in contrast with their results, however, neither protein was detected in the protoplast culture medium.

Similar Western blot analysis was carried out on the S30 fraction from protoplasts infected with mutant transcripts from pGM512, pGM524 and pGM512+524. The results (Figure 5.6) showed that while the two single mutants, pGM512 and pGM524, produced both the 48 and 58K proteins, the double mutant pGM512+524 produced only the 58K protein. Since the 48K protein is believed to be derived from the 95K primary translation product (see Figure 1), these results are consistent with in vitro translation data from the mutants which demonstrated that initiation of the 95K protein could occur from either the AUG at 512 or the

FIGURE 5.6.

Western blot analysis of the CPMV M RNA-encoded 48K and 58K proteins in infected protoplasts.

Protoplasts were electroporated with either virion B RNA (1ug/10^6protoplasts) plus (A) M RNA (1ug/10^6protoplasts),

or virion B RNA (1ug/10^6protoplasts) plus transcripts (6ug/10^6protoplasts) from:-

(B) pPMM2902.

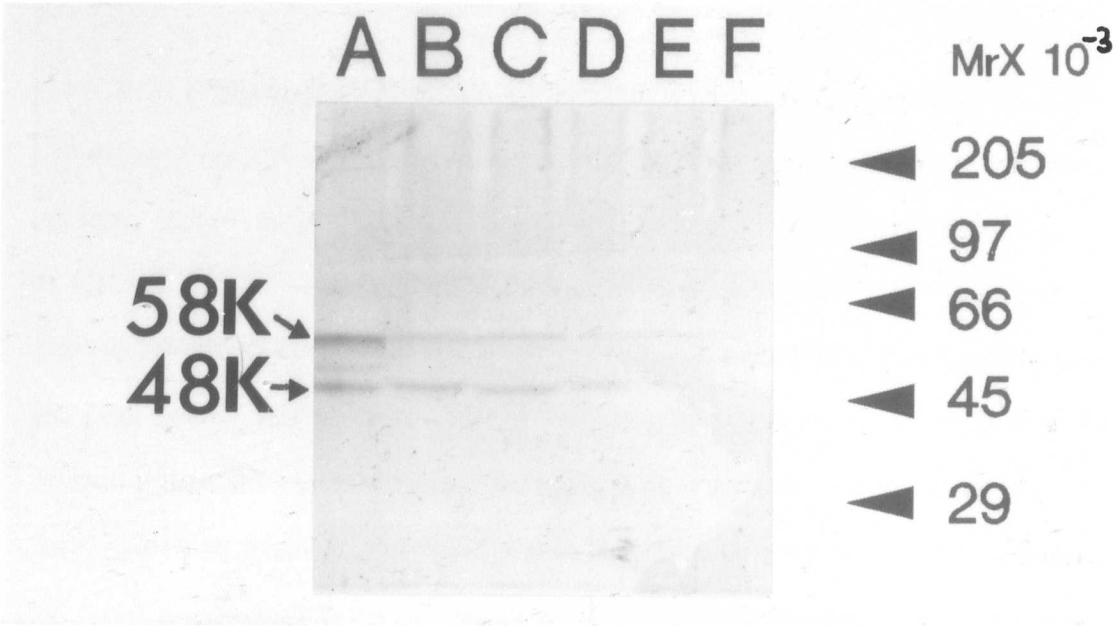
(C) pGM512.

(D) pGM524.

(E) pGM512+524.

or (F) B RNA alone.

The proteins have been separated on a 7.5% polyacrylamide/SDS gel. Each track was loaded with the protein extract from $2x10^5$ protoplasts.
The blots were probed with the 58/48K antiserum (see 2.2 G6). The size of protein markers used is indicated on the right hand side. The positions of the 58K and 48K proteins are marked on the left hand side.


A B C D E F
MrX 10-3
205
97
66
45
29
58K
48K

AUG at 524. Consistent with the results from in vitro translation, the "48K" protein directed by pGM512 transcripts migrates slightly ahead of that synthesised from wild-type and pGM524 transcripts (Figure 5.6, compare track C with tracks B and D).

5.7 Discussion.

The mechanism of translation of CPMV M RNA was investigated by making directed mutations in pPMM2902 and hence in the RNA transcribed from it in vitro.

Two mutants were produced to investigate the expression of the 105K protein; the first (pIM1) has a frame-shift downstream of the AUG at 161 and is similar to that of mutant pSPM5Δ*Bgl*II (Vos et al. 1984) and the second (pGM161) has an AGC codon in place of the AUG at position 161. Transcripts from both mutants had the same phenotype, directing the synthesis of the 95K and not the 105K protein in vitro and not being detectably biologically active in vivo. The in vitro translation data from pGM161 transcripts confirms the previous identification of the AUG at position 161 as the initiator for synthesis of the 105K protein (van Wezenbeek et al., 1983; Vos et al., 1984).

There are several possible explanations for the failure of transcripts from pGM161 and pIM1 to either replicate or produce coat protein antigen in protoplasts. The simplest is that the region of M RNA including sequences around position 161 and 189 is part of the region recognised by the B RNA-encoded replicase. The mutations in pGM161 and pIM1 could adversely affect these sequences thus preventing the transcripts from being replicated. An alternative possibility is that expression from the AUG at 161 is required for efficient multiplication of the M RNA in protoplasts. The 105K protein is translated from the AUG at 161, this protein being the precursor to the 58K protein and the coat

proteins. The 105K and/or 58K proteins may have an essential role in the replication of the M RNA. Alternatively, if the viral coat proteins are always produced by processing of the 105K protein rather than the 95K protein; failure to produce the 105K protein would thus prevent viral RNA encapsidation, leading to a failure of the viral RNA to accumulate (de Varennes and Maule, 1985). This possibility cannot be excluded since mutants with deletions in the coat protein region of the RNA fail to accumulate viral RNA efficiently (See Chapter 6) and thus appear to be deficient in replication.

Site-directed mutants were produced to investigate expression of the 95K protein. The in vitro translation data from the mutants pGM512, pGM524 and pGM512+524 demonstrate that either of the AUGs at positions 512 and 524 can act as initiation codons for synthesis of the 95K, protein at least in rabbit reticulocyte lysates. The fact that transcripts from the double mutant pGM512+524 can replicate and synthesise coat proteins shows that the 95K protein is not essential for CPMV multiplication in protoplasts.

The detection of both the 58K and 48K protein in the S30 from infected protoplasts provides direct evidence that the double initiation event which gives rise to the 105K and 95K proteins in vitro also operates in vivo. Results obtained with the mutants pGM512, pGM524 and pGM512+524 demonstrate that the 48K protein (and, by inference, the 95K protein) can be produced by initiation from either the AUG at 512 or the AUG at 524 in vivo, a result consistent with the in vitro translation data. The results also indicated that production of the the 48K protein is not necessary for the viral replication cycle in protoplasts, the products derived from the 105K protein (the 58K and the two coat proteins) being sufficient. The finding that the 48K protein is not essential in protoplasts is consistent with the proposed role of this protein in transport of virus particles or RNA throughout the plant (Rezelman et al., 1982; Wellink et al.,

1987a).

A further question raised from the results presented here is whether both the AUGs at positions 512 and 524 can be used to initiate synthesis of the 95K protein in wild-type M RNA. If we assume that the scanning model for translation is valid in the case of CPMV, ribosomes will encounter the AUG at 512 before the AUG at 524. Since the AUG at 512 clearly can act as an initiator, at least some of the 95K protein will start at this point. The question really is whether the AUG at 524 is used as an initiator when the AUG at 512 is present. The scanning model predicts that if the genuine initiator for protein synthesis is destroyed, initiation will occur at the next available AUG provided it is in a suitable context. This prediction has been confirmed in the case of the expression of the Early Region 1A (E1A) protein of human adenovirus 5 (Downey et al., 1984) and may well explain the initiation of 95K protein at position 524 that we observe in the mutant pGM512. Following on from this, one might expect initiation of protein synthesis to occur at the next in frame AUG in mutant pGM512+524. Close examination of Figure 5.4 track F reveals a novel protein of less than 95kDa encoded by pGM512+524 transcripts, possibly a result of initiation of protein synthesis at the AUG at 632 although there is no further evidence to confirm this.

For both AUGs at positions 512 and 524 to be used in the wild-type situation for expression of the 95K protein, the AUG at 512 would have to be a "leaky" initiation codon. This possibility is rendered less likely by the fact that the AUG at position 512 occurs in a context close to that regarded as optimal for efficient initiation (Kozak, 1986b; Lutcke et al., 1987). However, "leaky" initiation from an AUG in an apparently good context has recently been demonstrated in the case of influenza B virus (Williams and Lamb, 1989) so the possibility that both the AUGs at 512 and 524 are used cannot be excluded by this argument.

Data on the apparent sizes of the "95K" and "48K" proteins directed by

transcripts from pPMM2902, pGM512 and pGM524 indicates that the AUG at 512 is probably the one actually used on wild-type RNA. In contrast, direct evidence that both AUGs may be used in vitro comes from the observation that the 48K protein sometimes migrates as a closely spaced doublet in polyacrylamide gels (See Figure 1 in Franssen et al., 1982). However, there is, at present, no information regarding the N-terminal sequences of the two proteins.

Chapter 6: Investigation of the M RNA sequences required for replication by the B RNA-encoded polymerase.

6.1 Introduction.

The observation that cowpea mosaic virus B RNA is able to replicate in protoplasts in the absence of M RNA suggests that M RNA does not provide trans-acting functions necessary for replication, but only cis-acting regulatory sequences necessary for its own multiplication (Goldbach et al., 1980; Rezelman et al., 1982). The only regions of homology between M RNA and B RNA are at the termini (Davies et al., 1979; Stanley and van Kammen, 1979; Najarian and Bruening, 1980; Lomonossoff et al., 1982). Since both genome segments of CPMV are replicated by the same enzyme or enzyme system, these similar sequences might be involved in replicase recognition.

The importance of terminal sequences for replication has been demonstrated in several virus systems, defective interfering (DI) particles being particularly valuable tools for such studies. DI particles are deletion mutants of viruses primarily noted for their ability to interfere with the replication of homologous or closely related viruses. The deleted genomes are smaller than those of the parental virus and, although they need not retain coding information, they must contain sequences that serve as recognition signals for replication. Thus analysis of the nucleic acid from DI particles can be used to help identify such signals. Sequence analysis of Sindbis virus and one of its DI genomes, coupled with sequence analysis of other alphaviruses, enabled the identification of regions thought to be important for replication (Ou et al., 1981; 1983). The sequences essential for replication were then defined by deletion analysis of a full length cDNA clone of the DI RNA, the wild-type clone generating biologically active RNA after in vitro transcription. Analysis of deleted transcripts in vivo

demonstrated that only sequences at the termini are specifically required for replication and packaging of the mutant genomes (Levis et al., 1986).

Although DI RNAs are ubiquitous in association with animal viruses, it is only recently that a DI RNA associated with a plant virus infection has been fully characterised (Hillman et al., 1987). The DI RNA is associated with the tombusvirus tomato bushy stunt virus (TBSV) and attenuates TBSV-induced symptoms and depresses virus synthesis in whole plants. Sequence analysis showed the DI RNA to be a colinear deletion mutant of the helper virus genome containing a mosaic of virally derived fragments. The sequences required for replication were not however determined.

The sequences on the 3' termini of the BMV genomic RNAs which are required for replicase recognition have been extensively investigated in vitro. Each of the three genomic RNAs has a 3' tRNA-like structure. Analysis of mutant and wild-type transcripts corresponding to this region indicates that all signals necessary for minus strand initiation are present in the 3' terminal 134 nucleotides which comprise this tRNA-like structure (Dreher et al., 1984; Dreher and Hall, 1988). Using full-length infectious transcriptional clones of BMV, French and Ahlquist (1987) identified sequences on BMV RNA 3 required for it to be replicated in vivo by proteins encoded by RNAs 1 and 2. In addition to demonstrating that areas within both 5'- and 3'-terminal non-coding regions are required for replication, the experiments also demonstrate the requirement for internal sequences. The length of 3' non-coding sequence required for efficient RNA 3 accumulation in vivo was greater than that required in vitro for minus strand initiation. For normal replication or RNA stability, a physical separation of the terminal domains also appeared to be required. The requirement of a 150-base segment of the intercistronic noncoding region for efficient RNA 3 accumulation was unexpected but not unparalleled. A precedent for an internal replicase recognition site,

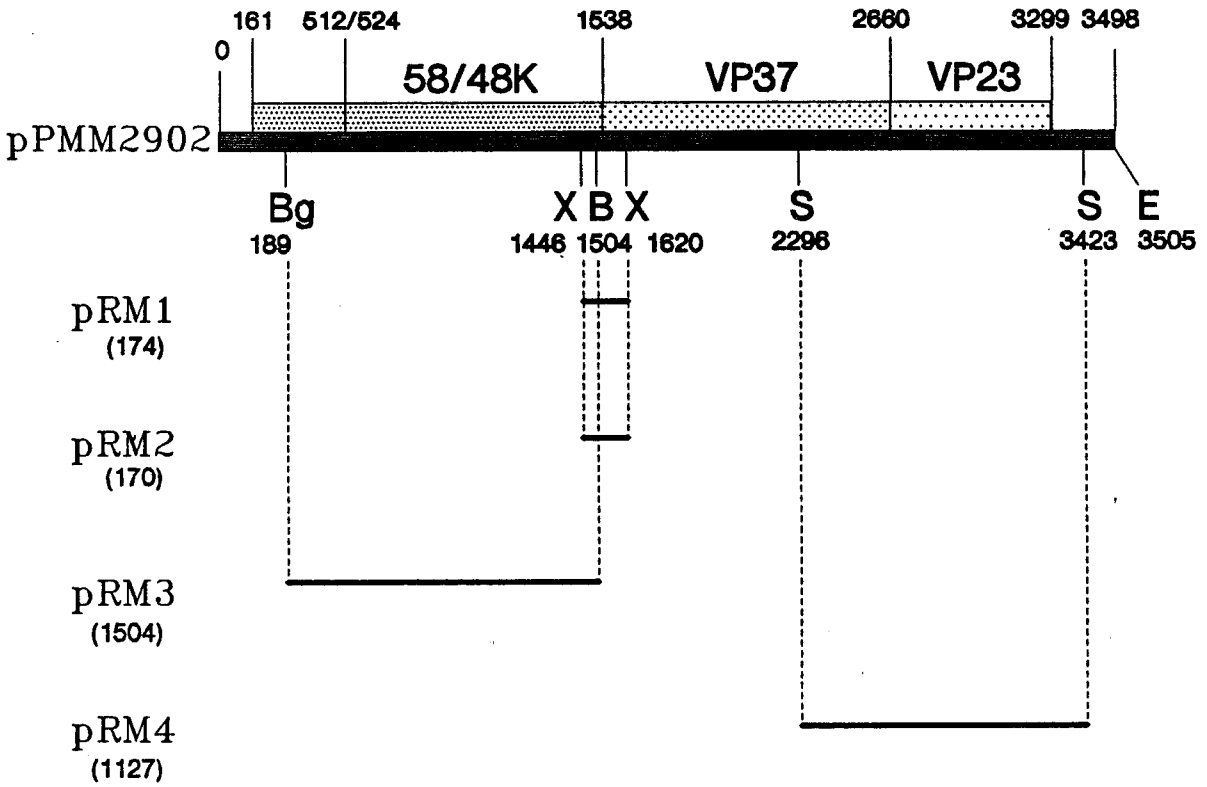


FIGURE 6.1

Schematic representation of the CPMV M RNA-specific portion of the pPMM2902 cDNA clone and deletion mutants pRM1-pRM4.

The dark shaded bar represents the M RNA cDNA and the genomic map is shown above. The nucleotide position corresponding to the N-terminal amino acid of each of the M RNA-encoded proteins is indicated by the numbers above the genomic map.
In mutants pRM1-pRM4 the regions indicated by black bars have been deleted. The size of each deletion (in nucleotides) is indicated in brackets.
Restriction sites in the wild-type sequence are Bg, BglII; X, XhoI; B, BamHI; S, SacI; E, EcoRI.
The nucleotide numbers below the cDNA show the position of each restriction site.

separated from its functional initiation site, exists in the single-stranded bacteriophage Qβ (Blumenthal and Carmichael, 1979, Meyer et al., 1984).

The work presented in this chapter is aimed at identifying those sequences in M RNA which are required for it to be replicated by B RNA-encoded polymerase. In order to do this, deletion mutants of M RNA were produced and tested for their productive replication in cowpea protoplasts coinoculated with B RNA. This work was possible owing to the availability of pPMM2902, an infectious transcriptional clone of CPMV M RNA (see Chapter 4). Deletions made in the cDNA clone result in transcripts lacking specific portions of the M RNA molecule. The results presented are preliminary but provide a basis for a more extensive study.

6.2 Production of deletion mutants.

Deletion mutants were produced by exploiting several of the restriction enzyme sites occurring only within the M RNA-specific region of pPMM2902. Figure 6.1 shows a restriction map of the M RNA cDNA and indicates the site of the deletion for each of the mutants produced. Mutant pRM3, containing a large deletion in the 5' half of the M RNA cDNA, was produced by cutting pPMM2902 with BamHI and BglII followed by ligation of the vector-containing fragment. The region deleted in pRM3 includes the AUG initiation codons at 512 and 524 and the deletion also induces a frame shift mutation, a UAA stop codon at position 1740 now being in frame with the AUG initiaton codon at 161.

Mutant pRM4, containing a large deletion in the 3' half of the M RNA cDNA, was produced by cutting pPMM2902 with SacI followed by religation of the vector-containing fragment. In mutant pRM4 the deletion extends into the 3' non-coding region and therefore includes the wild-type termination codon at position 3299. A UAA termination codon at 3439 is now in frame with the initiator AUGs.

Mutants pRM1 and pRM2 contain smaller internal deletions created by digestion

of pPMM2902 with XhoI, pRM1 being produced by direct religation of the vector containing fragment and pRM2 being produced after "filling-in" the protruding ends of the restricted vector before religation. The wild-type reading frame is retained in mutant pRM1 but lost in mutant pRM2. The "filling-in" of the XhoI sticky ends during the production of pRM2 inserts four base pairs and causes a frame shift mutation, a UGA stop codon at 1654 now being in frame with the initiation codons at 161, 512 and 524.

All deletion mutations were confirmed by restriction digest mapping.

6.3 In vitro transcription and in vitro translation of mutant transcripts.

Transcription reactions were performed on EcoRI linearised pRM1, pRM2, pRM3 and pRM4 plasmids to produce M RNA transcripts with specific deletions. To examine whether the open reading frames in the deletion mutants had been altered as predicted, in vitro transcripts from the four mutants and pPMM2902 were translated in reticulocyte lysates (Figure 6.2). As shown in the previous chapters, transcripts from pPMM2902 gave the 105K and 95K proteins characteristic of CPMV M RNA. The mutant transcripts all directed the synthesis of products that were shorter than the 105K and 95K proteins. The size of the products directed by the mutants is dependent on the size of the deletion and the effect of the deletion on the long open reading frame. Table 6.1 shows the predicted and apparent sizes of the protein products synthesized by the deletion mutants.

When transcripts from pRM1 are expressed in vitro, the apparent size of the proteins produced (as estimated after polyacrylamide gel electrophoresis), is approximately 10kDa less than the predicted size. This observation is however consistent with previous reports that electrophoretic mobilities of M-RNA encoded polyproteins only allow the determination of molecular weights which are at best

FIGURE 6.2.

<u>In vitro</u> translation products from wild-type and mutant transcripts of CPMV M RNA.

Products from the translation of transcripts from:-

(A) pPMM2902.

(B) pRM1.

(C) pRM2.

(D) pRM3.

(E) pRM4.

Products were analysed on a 10% polyacrylamide/SDS gel. The size of protein markers is indicated on the right hand side.

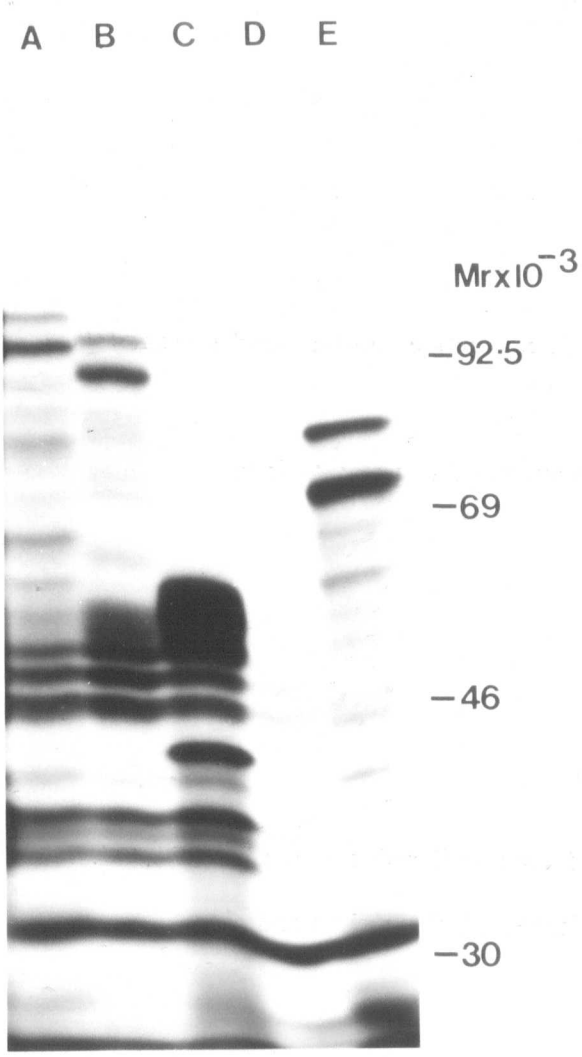

A
B
C
D
E
Mr x 10^-3
92·5
69
46
30

TABLE 6.1 Predicted and apparent sizes of in vitro translation products from wild-type and mutant transcripts of CPMV M RNA.

Name of clone	Translation initiates at[a]	Sequences deleted[b]	Translation terminates at	Predicted size of product(s)[c]	Apparent size of product[d]
pPMM2902	161, 512	none	UAA at 3299	116; 102	105; 95
pRM1	161, 512	1446-1620	UAA at 3299	110; 96	96; 87
pRM2	161, 512	1450-1621	UGA at 1654	50; 44	59; 43
pRM3	161	189-1504	UAA at 1740	10	ND.
pRM4	161, 512	2296-3423	UAA at 3439	81; 75	80; 72

a The second in-frame AUG on the long open reading frame of M RNA at position 512 is taken as the initiation site for the 95K protein (see Chapter 5).

b This information is also present in Figure 6.1.

c Predicted size of product (kDa) obtained by considering the effect of the deletions on the sequence of M RNA.

d Size of protein (K) as estimated after polyacrylamide gel electrophoresis. The value K represents apparent kDa.

NDNot determined.

a good approximation of the real size of the proteins (Goldbach and Van Kammen, 1985; eg. the 105K and 95K proteins have predicted molecular weights of 116 and 102kDa respectively). In contrast, the proteins directed by pRM2 and pRM4 have apparent molecular weights similar to those predicted. It is possible that the amino acids responsible for the aberrant migration of wild-type and pRM1 encoded proteins have been deleted from the pRM2 and pRM4 protein products, indeed the mutation in both pRM2 and pRM4 results in the deletion of the C-terminus of VP37 and all of VP23.

The predicted 10.6K product encoded by transcripts from pRM3 was not resolved by the electrophoresis system used.

In vitro translation of the deleted transcripts gave products of the expected size indicating that the reading frame in each of the mutants had been altered as predicted.

6.4 Electroporation of cowpea protoplasts with transcripts from pRM1 and pRM3.

Transcripts from pRM1 and pRM3 were chosen for the initial studies because the deletions did not effect the non-coding regions. Although mutants pRM1 and pRM2 contain similar deletions, pRM1 was favoured since in this mutant the reading frame is not affected.

6.4(A) Immunofluorescent assay.

When pRM1 and pRM3 transcripts were independently electroporated into protoplasts in the presence of purified virion B RNA, viral coat protein could not be detected by immunofluorescent assay. This result is not surprising for pRM3 transcripts since the coat proteins will not be synthesised owing to a frame shift mutation in the open reading frame. The reason for the failure to detect coat protein in protoplasts electroporated with transcripts from pRM1 is not

clear. The deletion does not affect VP23 but removes the cleavage site for the release of the 60K precursor to the coat proteins and the first twenty-one amino acids of VP37. However, the proteins that are synthesised (96K and 87K) should contain the remainder of VP37 and all of VP23. It is possible that, if synthesised in vivo, this uncleaved coat protein precursor is not antigenic or unstable and therefore not detected by the immunofluorescent assay.

6.4(B) Northern blot analysis to assess the replication of pRM1 transcripts in protoplasts.

Northern blot experiments indicate that pRM1 transcripts can replicate in cowpea protoplasts (Figure 6.3). A low level of hybridisation occurs in nucleic acids from protoplasts electroporated with pRM1 transcripts (Figure 6.3; Panel F), this low level being significant since no equivalent hybridisation occurs in the negative controls (Figure 6.3; Panels A,D,E). In addition to the mock electroporated and B RNA electroporated controls (Figure 6.3; Panels A and D), protoplasts were electroporated with transcripts from pIM1 (Figure 6.3; Panel E). The mutant pIM1 is discussed fully in Chapter 5 but is also included here since it provides an important negative control, transcripts from pIM1 apparently being unable to replicate in cowpea protoplasts.

M RNA accumulation in protoplasts electroporated with pRM1 transcripts is less than 1% of that found in protoplasts electroporated with wild-type transcripts. This reduced accumulation could be due to inefficient replication of the mutant transcripts or a result of the transcripts not being encapsidated (see discussion).

6.4(C) Northern blot analysis to assess the replication of pRM3 transcripts in protoplasts.

Transcripts from pPMM2902 and pRM3 were electroporated into protoplasts in the presence of virion B-RNA. Transcripts from pPMM2902 replicated in the

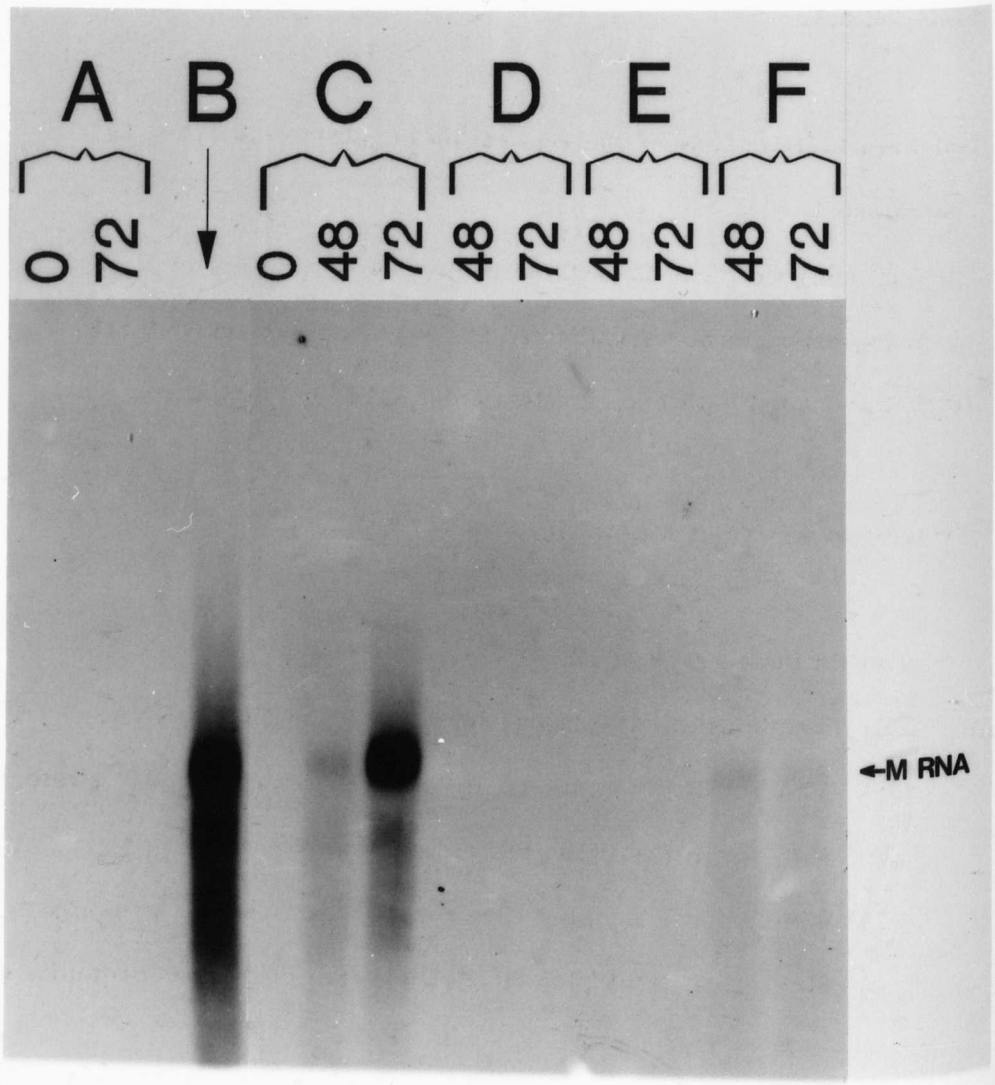

A
B
C
D
E
F
0
72
0
48
72
48
72
48
72
48
72
←M RNA

FIGURE 6.3.

Northern blot analysis of the replication of pRM1 transcripts in protoplasts.

Nucleic acids were extracted from protoplasts at times after electroporation, in hours, indicated at the tops of the tracks. Each track was loaded with nucleic acids from $1x10^5$ protoplasts.

Protoplasts were electroporated:-

(A) In the absence of transcripts.

(B) Sample as in (A) 72h but doped with 5ng CPMV RNA.

(C) With 1ug B RNA plus 1ug pPMM2902 transcripts/10^6 protoplasts.

(D) With 1ug B RNA/10^6 protoplasts.

(E) With 1ug B RNA plus 10ug pIM1 transcripts/10^6 protoplasts.

(F) With 1ug B RNA plus 10ug pRM1 transcripts/10^6 protoplasts.

The position of the M RNA is indicated.

protoplasts (Figure 6.4, Panel C) but replication of the mutant transcripts could not be detected by Northern blotting (Figure 6.4, Panel D).

Only 0.5ug of full length pRM3 transcript was electroporated per million protoplasts because, at the time of these experiments, little E.coli RNA polymerase was available. Electroporation with this amount of pPMM2902 transcript was shown in Chapter 4 to give infection levels of less than 8% by immunofluorescence. Consistent with this, an infection level of 3% was obtained after electroporation of pPMM2902 transcripts plus B RNA in this experiment. On previous occasions when more pPMM2902 transcript was electroporated, infection levels of up to 23% have been obtained (See Chapter 4, Table 4.3). Although replication of the wild-type transcripts was easily detected by Northern blotting (Figure 6.4, Panel C), the inability to detect mutant progeny RNA as a result of using sub-optimal conditions can not be ruled out. Indeed, when 1-2ug of full length pRM1 transcripts are electroporated per million protoplasts replication of the mutant transcripts is not detected by Northern blotting.

Transcripts from pRM3 contain a frame-shift and hence do not direct the synthesis of coat proteins. Any progeny RNA from pRM3 transcripts will therefore not be encapsidated. In order to determine whether the presence of coat proteins would have any effect on the accumulation of progeny pRM3 molecules, pRM3 transcripts were electroporated into protoplasts in the presence of virion B RNA and either M RNA or pPMM2902 transcripts. The pRM3 transcripts did not accumulate in the protoplasts in the presence of coat protein, no progeny RNA molecules of size equivalent to pRM3 transcripts being detected by Northern blotting (Figure 6.4, Panels E and F).

FIGURE 6.4.

Northern blot analysis of the replication of pRM3 transcripts in protoplasts.

Nucleic acids were extracted from protoplasts at times after electroporation, in hours, indicated at the tops of the tracks. Each track was loaded with nucleic acids from $1x10^5$ protoplasts.

Protoplasts were electroporated with:-

(A) Nucleic acids from mock electroporated protoplasts doped with 10ng CPMV RNA.

(B) 1ug B RNA plus 1ug M RNA/10^6 protoplasts.

(C) 1ug B RNA plus 0.5ug pPMM2902 transcripts/10^6 protoplasts.

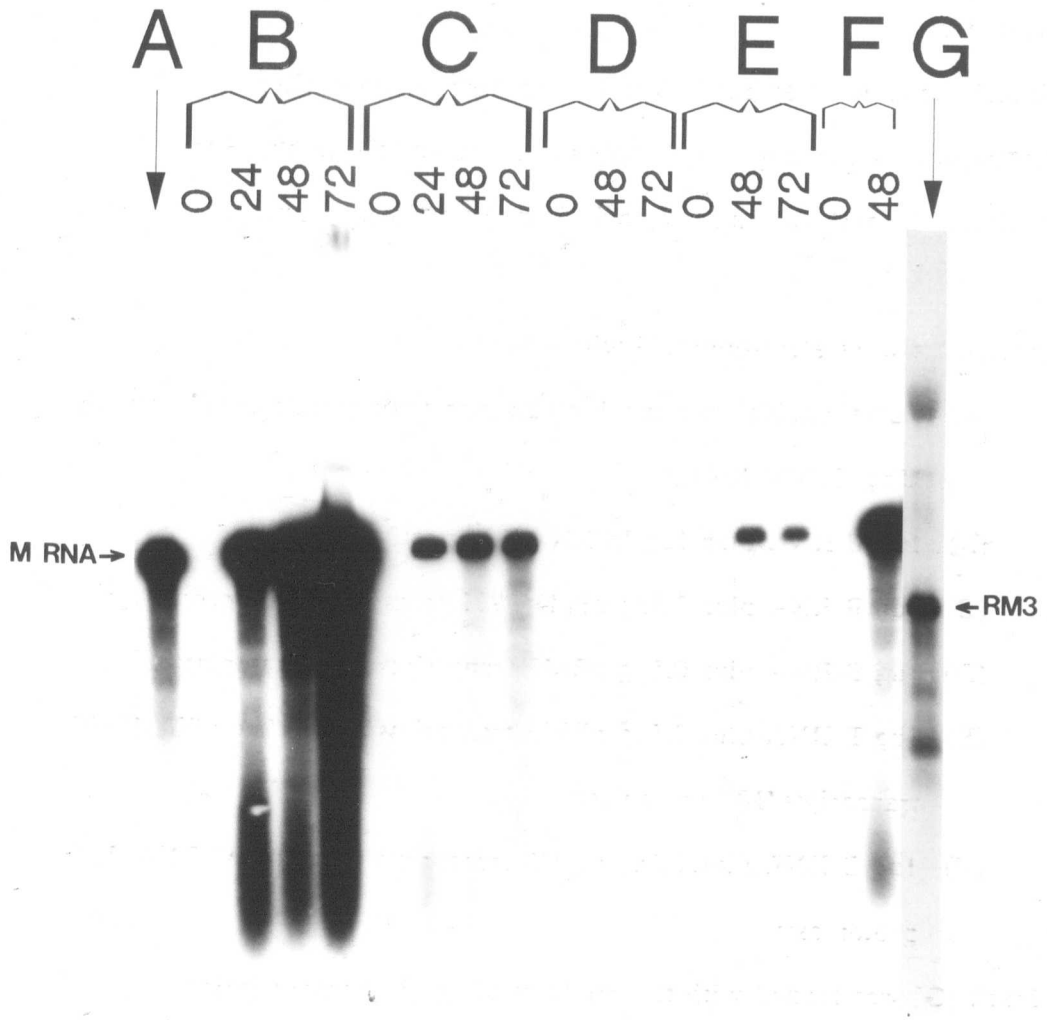
(D) 1ug B RNA plus 0.5ug pRM3 transcripts/10^6 protoplasts.

(E) 1ug B RNA plus 0.5ug pRM3 transcripts plus 0.5ug pPMM2902 transcripts/10^6 protoplasts.

(F) 1ug B RNA plus 0.5ug pRM3 transcripts plus 1ug M RNA/10^6 protoplasts.

Track (G) was loaded with the products of <u>in vitro</u> transcription directed from 2ng of linear pRM3 DNA templates. The position of the full length pRM3 transcripts is indicated on the right hand side.
The position of M RNA is indicated on the left hand side.

The slight hybridisation above the M-RNA corresponds to B-RNA. This cross hybridisation occurred since a genomic length M-RNA probe was used and there are regions of homology at the termini of the genomic RNAs.


A B C D E F G
0 24 48 72 0 24 48 72 0 48 72 0 48 72 0 48
M RNA→
←RM3

6.5 Discussion.

By exploiting the restriction enzyme sites in pPMM2902 deletion mutants were generated quickly and easily. The open reading frame of each mutant was examined by translation of mutant transcripts in reticulocyte lysates, the apparent size of the proteins being consistent with the predicted size, confirming the authenticity of the deletion mutations.

Electroporation of cowpea protoplasts with transcripts lacking specific portions of the M-RNA indicated that nucleotides 1446-1620 are not essential for replication. These transcripts from pRM1 replicate in cowpea protoplasts but their accumulation is markedly reduced when compared with wild-type transcripts. This reduced accumulation may reflect inefficient replication of the mutant transcripts or be a result of lack of transcript encapsidation. Unencapsidated B RNA is unstable in vivo (De Varennes and Maule, 1985) and the same probably applies to M RNA. The idea that a mutation in M RNA preventing coat protein production will reduce RNA accumulation in protoplasts, is supported by the analysis of the mutant pPMM29. pPMM29 is identical to pPMM2902 except that a single base pair is deleted at position 2419 of the M RNA-specific DNA, resulting in a frame shift mutation within VP23 (G. P. Lomonossoff, personal communication). In cowpea protoplasts, transcripts from pPMM29 show a marked reduction in accumulation (J. Rohll, personal communication). Although it can not be ruled out that the small alteration in the RNA of pPMM29 does interfere with replication, it is more probable that the reduced accumulation of pPMM29 transcripts results from lack of encapsidation.

Assuming that transcripts from pRM1 do not encapsidate, the reduced accumulation of pRM1 transcripts in protoplasts is probably a result of this rather than inefficient replication. During this work, coat proteins were not detected by immunofluorescent assay of protoplasts electroporated with

transcripts from pRM1. However, in a recent report, Wellink and Van Kammen (1989) could apparently detect some coat protein in protoplasts infected with transcripts having an identical mutation to that of pRM1. The reason for this discrepancy is not clear but is possibly a result of differences in anti-CPMV serum used during the immunofluorescent assays. However, even if expressed in vivo, transcripts from pRM1 are probably not encapsidated since the deletion effects the VP37 and the proteolytic cleavage site used to generate the precursor to the coat proteins.

The replication of transcripts from pRM3 bearing a deletion within the 58/48K coding region (from nucleotide 189-1504) could not be detected in the protoplast system. It is tempting to link this observation with the inability of transcripts from pIM1 and pGM161 to replication in vivo. Transcripts from pIM1 and pGM161 are discussed in Chapter 5 and contain mutations around nucleotides 189 and 161 respectively. The results obtained with transcripts from pRM3, pIM1 and pGM161 indicate that cis-acting regulatory sequences may not be confined to the non-translated regions but extend downstream of the first initiation codon, the region around nucleotides 161 and 189 being particularly sensitive to mutation.

The aim of this work was to investigate those sequences on M RNA required for it to be replicated by B RNA. To achieve this, deletion mutants of pPMM2902 were produced. Although CPMV RNA is not stable in vivo, preliminary results indicate that unencapsidated replicating mutants (such as pPM29 and pRM1) may possibly be distinguished from those mutants (such as pIM1, pRM3 and pGM161) which are unable to replicate efficiently, if at all.

If transcripts from pRM1 do accumulate poorly as a result of lack of encapsidation, encapsidated progeny pRM1 molecules should accumulate more efficiently. To investigate whether pRM1 transcripts contain sequences required for encapsidation and, if so, whether encapsidated transcripts accumulate more

efficiently, experiments were designed in which coat proteins would be provided in trans.

Electroporation of protoplasts with mutant transcript plus B RNA and M RNA will result in encapsidated transcripts (in addition to encapsidated virion RNAs) provided that the mutant transcripts contain sequences required for replication and encapsidation. Transcript progeny may then be detected (in the presence of virion M RNA progeny) if the deletion mutation is sufficient to resolve mutant RNA from the full-length M RNA by Northern blotting. (This approach was used for pRM3 transcripts but progeny RNA molecules were still not detected). For deletion mutants such as pRM1 that contain only a small deletion, transcripts may be selectively detected in the presence of M RNA if they contain a unique marker sequence, Northern blotting and probing for the marker sequence detecting only the mutant RNA. A marker sequence (from nucleotides 1928-2150 of pCa24, Delseny and Hull, 1983) was cloned into the XhoI site of pRM1 giving pRM1.ICA. Unfortunately, time did not permit an analysis of transcripts from pRM1.ICA.

CHAPTER 7:General discussion.

7.1 Traditional genetics.

In this thesis the translation and replication of CPMV was investigated using both traditional and directed mutants. First, the nitrous acid-induced mutant of CPMV known as 8-14 was further characterised. The lesion(s) in 8-14 was found not to prevent translation of the genome or the first proteolytic cleavage of the B RNA-encoded polyprotein. The defect is probably at the level of genome replication. The mutant phenotype could result from a mutation in any of the CPMV-encoded proteins thought to have a role in replication, the mutation possibly effecting the 87K or 54K proteins within the replication complex or the VPg. Alternatively, the lesion in 8-14 could disrupt a feature of the RNA required for replicase recognition.

The genetic lesion responsible for the ts phenotype of 8-14 was not located using the technique of two dimensional RNA fingerprinting. However, the number of induced genetic alterations in the 8-14 genome was shown to be less than the variation occuring between two wild-type isolates of CPMV as a result of evolution. To define the genetic lesion, a sequence analysis of mutant and revertant genomes could be carried out. This would, however, be laborious and would not necessarily define a single genetic lesion for the 8-14 phenotype. The physiological lesion in 8-14 was also thought to be difficult to define since any of several possible mutations could cause a replication defective phenotype. An analysis of proteins involved in 8-14 replication would be especially troublesome since there is no in vitro replication system for CPMV and the role of CPMV-encoded proteins in replication is poorly understood.

The best approach to further investigations of 8-14 would probably be to

produce and analyse full-length biologically-active cDNA clones of mutant and revertant genomic RNAs. Transcriptional clones of 8-14 M RNA and B RNA would greatly facilitate studies of 8-14, eliminating the problems of reversion and poor propagation in mutant virus-infected plants by providing a plentiful and consistent source of 8-14 RNA. In addition, full length cDNAs of mutant and revertant B RNAs could be used to locate the genetic lesion in 8-14 by creating mutant-revertant hybrid genomes and testing in vitro generated transcripts for temperature sensitivity. This approach would allow location of a region of the 8-14 B RNA responsible for the ts phenotype, the genetic lesion then being defined by sequence analysis of this region from mutant and revertant genomes.

7.2 Reverse genetics.

7.2(A) Translation of cowpea mosaic virus M RNA.

The availability of a full-length infectious clone of CPMV M RNA, from which infectious RNA can be generated by in vitro transcription, allowed the isolation of mutants by reverse genetics. The translation of CPMV M RNA was investigated by directing mutations to each of the presumptive translation initiators at nucleotide positions 161, 512 and 524, and by mutating the initiators at 512 and 524 together. Analysis of the mutant transcripts in reticulocyte lysates showed the CPMV M RNA-encoded 105K and 95K proteins to be produced as a result of independent intiation. The 105K protein initiates at the AUG at 161 and the 95K protein initiates at the AUG at either 512 or 524, initiation at 524 probably only occurring in the absence of an AUG at 512.

In CPMV-infected protoplasts the 58K and 48K proteins were detected. Since the 58K and 48K proteins are the N-terminal cleavage products of the 105K and 95K proteins respectively, this demonstrates that at least two separate initiation events occur on M RNA when CPMV infects cowpea mesophyll protoplasts. The detection of the 48K and 58K proteins in protoplasts infected with mutant

M RNAs showed that the 48K protein may initiate in vivo at the AUG at 512 or 524 but once again initiation at only 512 probably occurs in the wild-type situation.

The results indicate that alternate initiation codons within the same reading frame of M RNA are used in vivo. Although most eukaryotic messenger RNAs are functionally monocistronic, certain other viral mRNAs have been shown to synthesize two separately initiated proteins (reviewed by Kozak, 1986a). The scanning model for initiation of translation (Kozak, 1978; 1981; 1986b and 1989) can be applied to explain the production of two proteins from one mRNA. The scanning model postulates that a 40S ribosomal subunit binds initially at the 5' end of a mRNA and migrates until it reaches the first AUG triplet, which will serve as the unique site of initiation if the AUG occurs in the optimal context of CACCAUGG for animals (Kozak, 1986) or AACAAUGG for plants (Lutcke et al., 1987). An AUG codon is said to be in a good context if the key positions at -3 and +4 conform well to the consensus. However, initiation at an internal AUG can occur either if translation from the first AUG terminates before reaching the internal AUG; or, if the first AUG occurs in a suboptimal context some ribosomes will initiate there and others at the downstream AUG, this mechanism being known as leaky scanning.

The first difficulty encountered when applying the scanning model to the expression of CPMV M RNA is the presence of VPg covalently bound to the 5' end of the RNA. Since the VPg is not unlinked from the RNA during incubation in reticulocyte lysates (De Varennes et al.,1986), the VPg presumably presents a barrier to the initial binding of 40s ribosomes. Assuming that ribosomes do bind and scan along from the 5' end of the M-RNA, the first AUG encountered at 115 is in a poor context and in frame with a stop codon at position 175. No evidence exists for the expression of this small open reading frame. Initiation from the

AUG at 161 must therefore occur as a result of leaky scanning of the AUG at 115. Expression from the downstream AUGs at 512 or 524 can also be explained by the leaky scanning mechanism since the AUG at 161 lies in a poor context. It is also possible that any ribosomes that do initiate at position 115 and terminate at 175 may then reinitiate at 512 or 524. The AUG at 512 is in a better context than that at 524 and is probably favoured, or used solely, in the wild-type situation. The expression of the CPMV M-RNA may therefore be explained theoretically by applying both termination-reinitiation and leaky scanning models.

It has recently been proposed that some messenger RNAs are expressed by an internal initiation mechanism which is not dependent on ribosomes binding to, and scanning from, the 5' end (Pelletier and Sonenberg, 1989; Jang et al., 1989). The exact mechanism of the process, and the nature of sequences required, is not known. The possibility that the CPMV M RNA-encoded 95K protein is translated by ribosomes which bind to an internal region instead of scanning from the 5' end, has not been investigated to date.

7.2(B) Replication of cowpea mosaic virus M RNA.

Replication of CPMV in protoplasts was found to be independent of an initiator at positions 512 and 524 and hence production of the 95K or 48K proteins. The 48K and 58K proteins are thought to have a function in virus transport throughout an infected plant (Wellink and Van Kammen, 1989). The dispensibility of the 48K protein in the single cell protoplast system is consistent with this proposed role.

Alteration of the AUG at 161 prevented the accumulation of M RNA and M RNA-encoded proteins in protoplasts. Two possible explanation for this are either that expression from the AUG at 161 to give the 105K is a requirement for replication, or replication is directly affected as a result of the nucleotide alterations.

Several deletions were made in the M RNA cDNA in order to determine which sequences were essential for replication by the B RNA-encoded polymerase. Mutant pRM1, having a deletion at the 48/58K-60K cleavage site, replicated in cowpea protoplasts but the RNA accumulation was markedly reduced compared with the wild-type transcripts. This indicates that the deleted region (between nucleotdes 1446 and 1620) contains sequences which are probably not required for replicase recognition. It was not determined whether the reduction in accumulation of pRM1 was mainly a result of lack of transcript encapsidation or reduced replicability but the former explanation seems the most likely. Data from pRM1 and other deletion mutants (J. Rohll, unpublished observations) indicates that mutations which prevent encapsidation will have a drastic effect on the detection of progeny RNA molecules in infected protoplasts. This situation contrasts to that of brome mosaic virus, amplification of the RNA of deletion mutants being independent of encapsidation (French and Ahlquist, 1987). In spite of this problem, a replicating but non-encapsidated CPMV M RNA mutant such as pRM1 can be distinguished from mutants thought to be defective in replication such as pIM1, pGM161 and pRM3. Data from these latter three mutants shows the region of M RNA around nucleotides 161 and 189 to be particularly sensitive to mutation, transcripts having alterations in this region not accumulating in protoplasts. This region of M RNA may therefore contain signals required for replicase recognition.

The instability of unencapsidated CPMV RNA in protoplasts may be overcome by providing the coat proteins in trans, providing the mutant RNA molecules retain any sequences required for encapsidation. Such experiments involve electroporation of protoplasts with deleted M RNA in the presence of wild-type M RNA. Detection of progeny mutant RNA molecules is then dependent on the deletion in M RNA being big enough to allow wild-type and mutant M RNAs to be

resolved by Northern blotting or the mutant RNA carrying a specific marker sequence. These experiments may, however, be complicated by competition effects as wild-type and mutant RNAs compete for replicative machinery.

A different approach to providing coat protein in trans is to produce transgenic plants expressing VP37 and VP23. Since it is not possible to regenerate cowpeas from callus, transgenic tobacco would have to be produced. Although tobacco is not a host for CPMV, the virus does replicate efficiently in isolated protoplasts (Huber et al., 1977). Provided that the coat proteins, expressed in the transgenic tobacco protoplasts, were able to encapsidate virion RNAs, M RNA molecules with mutations effecting encapsidation could then be analysed for replication and encapsidation.

In this thesis some aspects of cowpea mosaic virus translation and replication were successfully investigated by isolating and characterising directed mutants of CPMV M RNA.

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